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The Science & Business of Biopharmaceuticals
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# A Renaissance in Biomanufacturing: The Art of Purification

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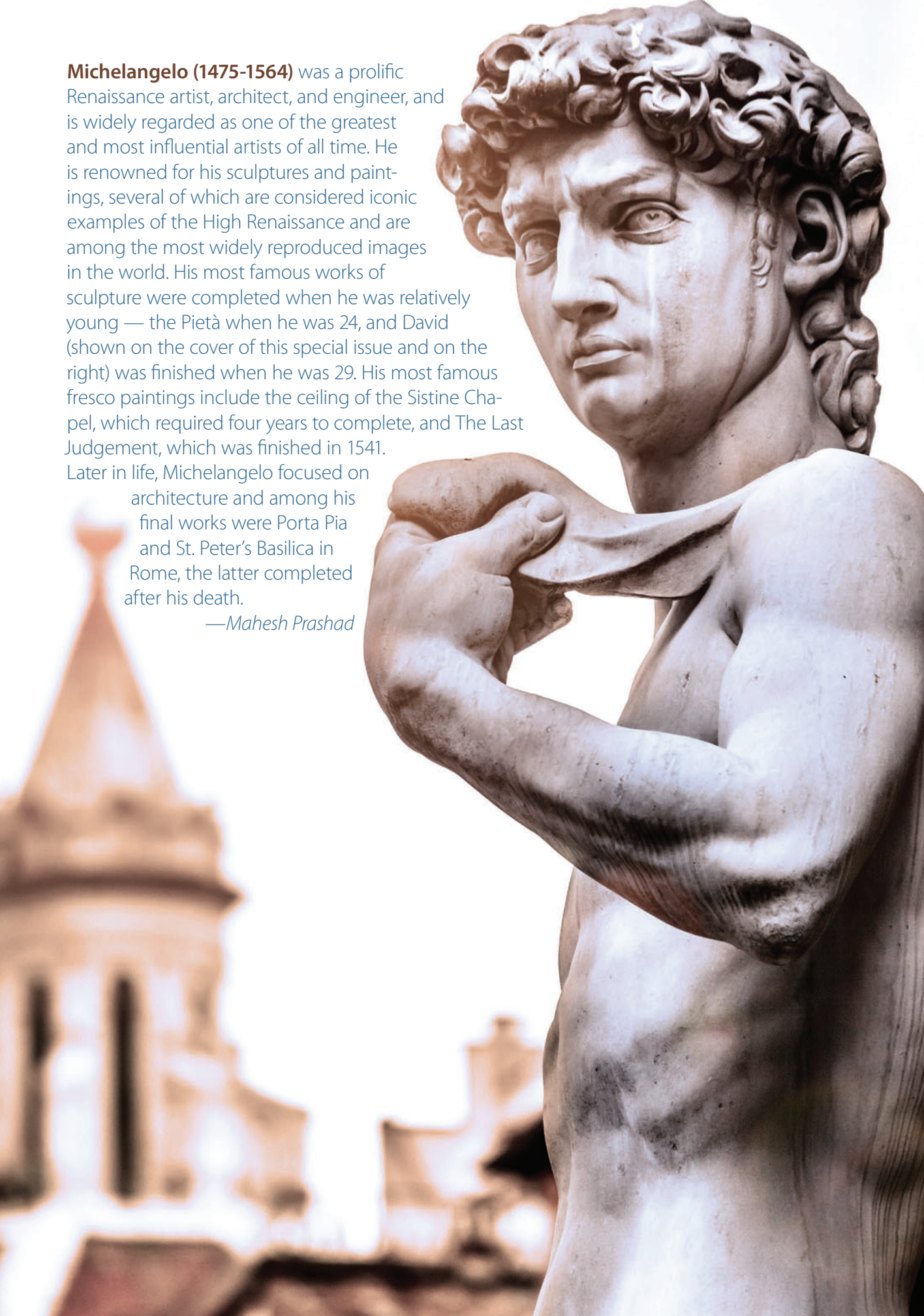
**Michelangelo (1475-1564)** was a prolific Renaissance artist, architect, and engineer, and is widely regarded as one of the greatest and most influential artists of all time. He is renowned for his sculptures and paintings, several of which are considered iconic examples of the High Renaissance and are among the most widely reproduced images in the world. His most famous works of sculpture were completed when he was relatively young — the Pietà when he was 24, and David (shown on the cover of this special issue and on the right) was finished when he was 29. His most famous fresco paintings include the ceiling of the Sistine Chapel, which required four years to complete, and The Last Judgement, which was finished in 1541. Later in life, Michelangelo focused on architecture and among his final works were Porta Pia and St. Peter's Basilica in Rome, the latter completed after his death.

*—Mahesh Prashad*



Supplement to:
BioPharm INTERNATIONAL
The Science & Business of Biopharmaceuticals



# Contents

## The Art of Purification



*This supplement to* BioPharm International *presents original articles as well as articles published in previous issues of* BioPharm International.

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# A Renaissance in Biomanufacturing: The Art of Purification

UWE GOTTSCHALK

The Renaissance sculptor Michelangelo described his work by taking blocks of marble and freeing a form already contained within by removing all that did not belong in the masterpiece. The same principles apply in downstream processing, where holistic concepts are used to remove everything that does not belong in the drug substance, and it helps to reframe this task by focusing on the contaminants rather than the product.

The Renaissance was a time of great innovation in Europe, a time when new ideas spread and took hold, and accepted paradigms were challenged. In the biomanufacturing industry, innovation is required. We need to adapt to a changing commercial environment shaped by diverse forces including the demands of the market, which try to drive down costs; the emergence of new industry players, particularly those seeking to profit from generics; and, of course, the regulatory authorities, which apply ever more stringent quality requirements to ensure patient safety. As the pincers close around us, we need to innovate to move forward, to take advantage of new technologies, processes, and strategies. We need to negotiate the changing landscape of our industry by thinking smarter and using new ideas to create new opportunities.

In seeking ways to improve our fortune, it is tempting to reach for the low-hanging fruit and thus make small and incremental improvements to our processes. But most of the easy pickings are already gone. We must look further than before and shine the light of

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change into every corner of biomanufacturing, no matter how well established and safe we feel the paradigms may be. Perhaps the best example to illustrate this change is polishing, the final phase of downstream processing, which is essential to remove impurities, and contaminants, and especially for the clearance of pathogenic agents such as endogenous and adventitious viruses.

Today, technology platforms are implemented early on in process development, and at least two orthogonal steps are assigned for the robust and efficient clearance of impurities and contaminants, ensuring that manufacturing processes meet the specifications established during validation. We need to harness the power of innovation to improve the efficiency and robustness of polishing without driving up the cost of goods.

Recent innovations in polishing that have started to address these goals include the use of membrane chromatography that can target all critical contaminants under a wide range of process conditions, but boost productivity at the same time; and the development of novel dead-end filters with tapered pores to increase virus retention without blocking. These developments come with new, integrated and (if appropriate) single-use concepts that meet the requirements of modern process trains.

This special supplement of *BioPharm International* collects together a number of groundbreaking articles by authors at the cutting edge of downstream processing, focusing on polishing and virus removal. The supplement begins with a report by Nathalie Frau et al. on the development of a new scale-down device for polishing and virus removal. This is complemented by an article by Min Lin and colleagues describing the development of a salt-tolerant membrane adsorber that can be used for polishing during the production of complex proteins in high-conductivity feed streams. Another report by Yun (Kenneth) Kang and colleagues outlines a novel approach to antibody polishing involving a combination of high-throughput screening and design of experiments optimization to establish a salt-tolerant interaction chromatography step for the efficient clearance of impurities from four antibodies under high-conductivity conditions. The final article by Thom et al. highlights the exciting development of a new high-throughput parvovirus-retentive membrane with an optional adsorptive prefilter as an integrated concept. These research articles are followed by a broader outlook discussing the future of downstream processing to demonstrate the need for continuous innovation in an industry that is running to stand still. The supplement closes with a short interview about the options of single-use technology in protein purification.

**Fortune favors the brave, and thus to ensure future progress, we need to prepare courageously for a commercial environment where change and adaptation is a way of life. We need to strive to be better, but also to be smarter.**

Fortune favors the brave, and thus to ensure future progress, we need to prepare courageously for a commercial environment where change and adaptation is a way of life. We need to strive to be better, but also to be smarter. Like the Renaissance masters that came before, we must not resist this season of change but instead we must embrace it! **BP**

# A New Scale-Down Membrane Adsorber Device for Process Development and Validation

Anion exchange membrane chromatography (AEX) is an attractive alternative to flow-through anion exchange column chromatography. Replacing AEX column chromatography with AEX membrane chromatography provides similar output but at a much higher load density, usually greater than 10 kg/L of membrane. The commercially available scale-down model, Sartobind nano, which has a 1 mL membrane volume, requires a significant amount of material for process development and validation whereas a relatively small amount of material is typically available during early clinical development. To overcome this limitation, an ultra scale-down device, Sartobind pico, was developed to reduce material consumption and validation cost. In this article, the development of the new ultra scale-down device is detailed and scalability to Sartobind nano and to a large-scale capsule are demonstrated. Studies using model proteins and industrially relevant monoclonal antibody feedstock are described. The new ultra scale-down device, Sartobind pico, enables process development, characterization, and validation with scalability to large-scale membrane chromatography devices while reducing sample consumption, time, and cost.

NATHALIE FRAU, MARTIN LEUTHOLD, AMIT MEHTA, KOME (KEVIN) SHOMGLIN, AND RENE FABER

Anion-exchange (AEX) membrane chromatography is an attractive technology for monoclonal antibody (mAb) purification because of advantages such as elimination of column packing and unpacking, higher throughput, smaller plant footprint, and considerably less buffer consumption. Compared with AEX resins, which are typically loaded to approximately 100 g/L, AEX membranes can provide orders of magnitude higher loading capacity in flow-through mode with adequate impurity removal. For example, Zhou et al. reported greater than 3000 g/m$^2$ or 10.9 kg/L load capacity with > 5 log reduction value (LRV) for four different model viruses (1). In another study, Zhou et al. showed that a similar LRV for X-MuLV could be obtained at a load capacity of 13 kg/L and at flow rate of 600 cm/hr (2). Glynn et al. recently described the evolution of Pfizer's antibody purification process from three columns to two by replacing the resin-based AEX chromatography step with a membrane adsorber and increasing the load capacity of this step by a factor of 100 (3). The removal of process-related impurities with AEX membrane adsorbers at high load capacity and high flow rate has also been published by Arunakumari et al. (4). Lately, the authors demonstrated virus removal by membrane adsorbers with a LRV greater than 4.5 and 4.4 for X-MuLV and MMV, respectively, at 20 kg/L mAb load capacity (5). Mehta et al. showed that purity and product quality comparable to traditional three-column affinity processes can be achieved with a novel process using a nonaffinity capture step and membrane-based technologies such as AEX membrane adsorbers and high performance tangential flow filtration (6).

It is thus well documented in the literature that an AEX membrane adsorber is a powerful alternative to column chromatography and can facilitate development of new purification strategies for downstream processing in the biopharmaceutical industry (7). However, the high load capacity achieved with membrane adsorbers in the flow-through mode implies the need for a significant amount of material for process development with laboratory-scale devices. For example, a load capacity of 10 kg/L means that 10 g of material is required for each experiment with a 1 mL laboratory-scale device.

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PEER-REVIEWED
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High material consumption can be a limiting factor, particularly during early stages of drug development where relatively small amount of material is typically available. Reducing the virus validation cost by minimizing the amount of virus spike required is also of significant interest.

To overcome these limitations, a new ultra scale-down membrane adsorber device, Sartobind pico (Sartorius Stedim Biotech GmbH, Göttingen, Germany), with a membrane volume of 0.08 mL has been developed. The 12.5-fold lower membrane volume than the current laboratory-scale device, 1 mL Sartobind Nano, significantly minimizes feedstock and virus spike requirements for development, characterization, and validation studies. The performance of this device was evaluated using model molecules and industrially relevant mAb feedstock and was compared with the current scale-down device, Sartobind nano. Data demonstrating the scalability of the new ultra scale-down device to a manufacturing-scale device are also presented.

## MATERIALS AND METHODS

### Devices

Sartobind pico, the new scale-down device was provided by Sartorius Stedim Biotech GmbH, Göttingen, Germany. The device consists of 15 membrane layers with polypropylene sealing rings every 3 layers, and is assembled into a molded polypropylene housing with luer lock connectors to enable easy connection to a liquid chromatography system (see **Figures 1** and **2**). The bed height of 4 mm is similar across the entire Sartobind SingleSep family and the frontal surface area of 20 mm² gives pico a membrane volume of 0.08 mL. Sartobind nano, (Sartorius Stedim Biotech GmbH, Göttingen, Germany) with 15 layers, 36.4 cm² total surface area, and 1 mL membrane volume was used as a reference device (see **Figure 1**). The Sartobind nano has a radial flow and is constructed in the same way as process scale SingleSep capsules, which assures direct scalability to manufacturing scale capsules (7–11). The key attributes of Sartobind pico and Sartobind nano are summarized in **Table I**.

The Sartobind SingleSep 10″ capsule with a membrane volume

**Table I:** Key attributes of Sartobind pico and Sartobind nano.

| | Sartobind nano | Sartobind pico |
|---|---|---|
| Bed height (mm) | 4 | 4 |
| Membrane volume (mL) | 1 | 0.08 |
| Housing materials | Polypropylene | Polypropylene |
| Connectors | Luer-Lock | Luer-Lock |
| Flow path | Radial | Axial |

**Figure 1:** A. Sartobind pico 0.08 mL. B. Sartobind nano 1 mL.

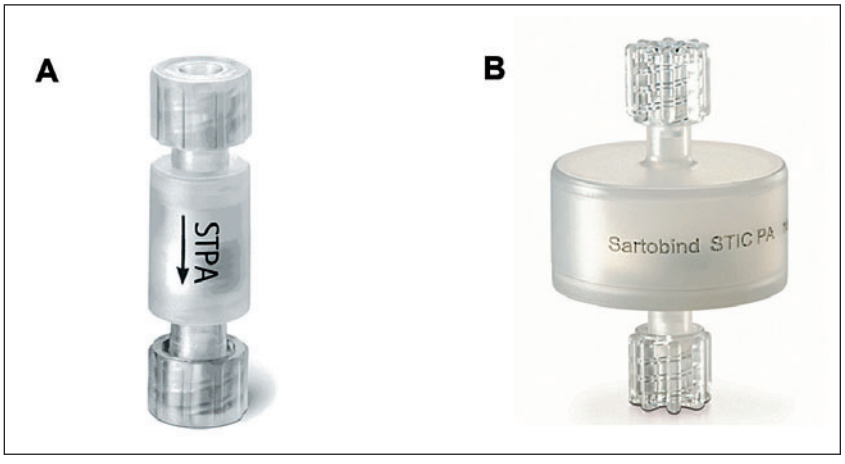
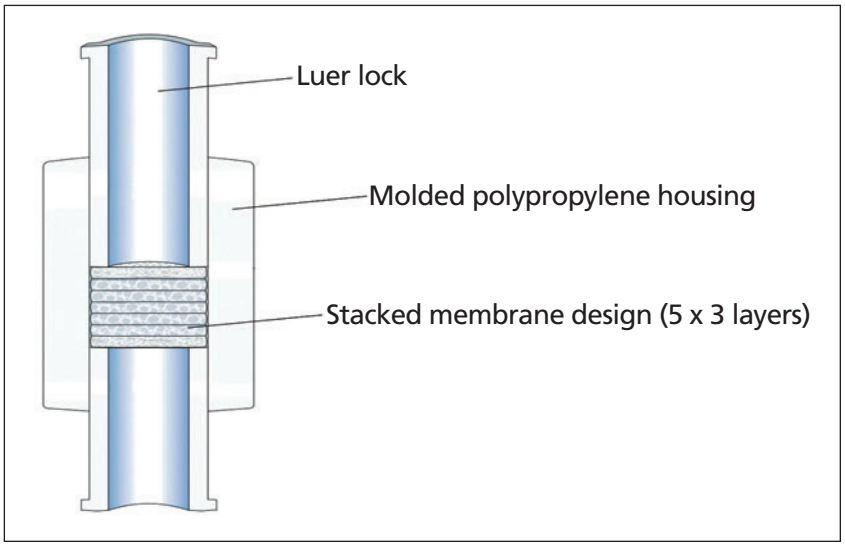



**Figure 2:** Sartobind pico device design.




ALL FIGURES ARE COURTESY OF THE AUTHORS

**Figure 3:** Normalized flow (MV/min) for Sartobind pico and nano devices as a function of inlet pressure. MV is membrane volume.

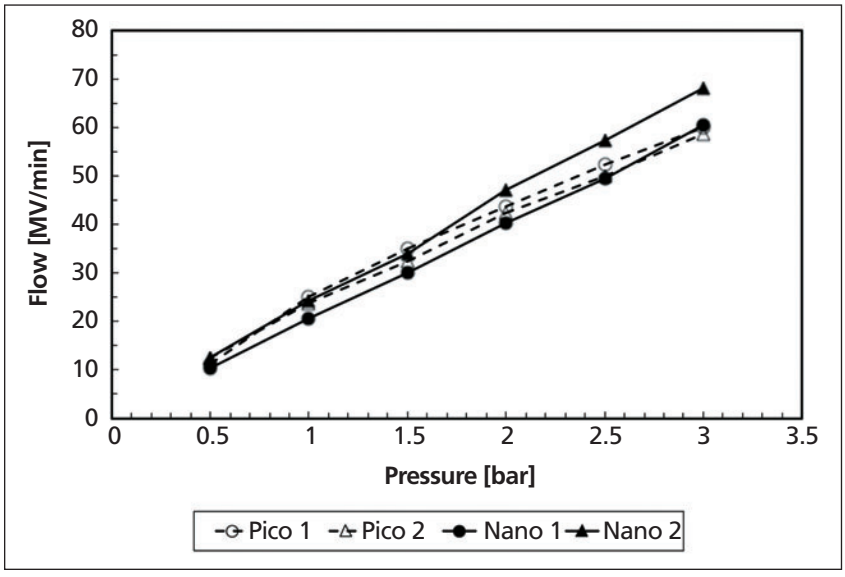
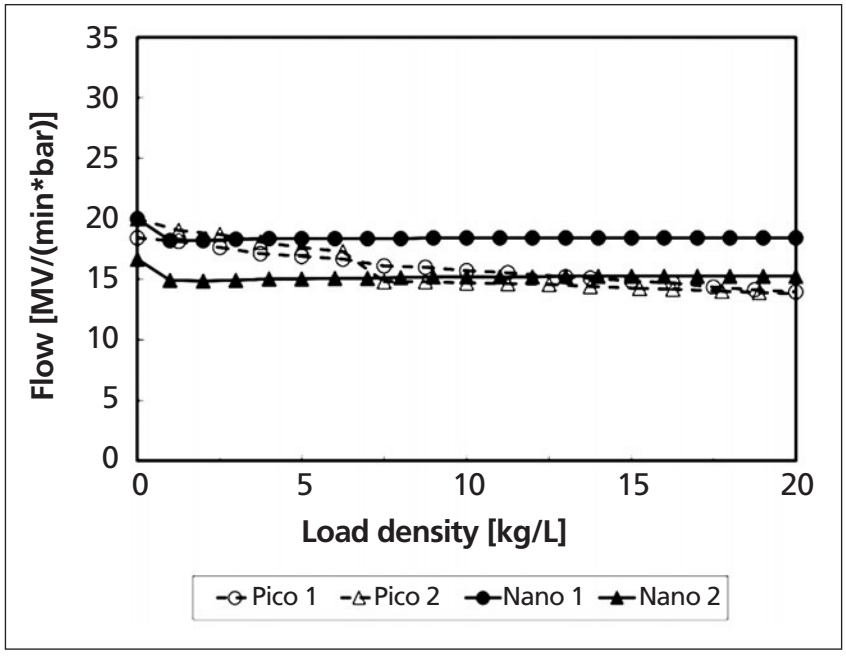



**Figure 4:** Filtrate flow rate at 3 bar constant initial pressure during loading of 20 g/L γ-globulin protein solution. MV is membrane volume.




of 180 mL was used to further confirm scalability. The devices were assembled with a salt tolerant AEX membrane, Sartobind STIC PA, consisting of a polyallylamine ligand covalently coupled to the cellulose membrane matrix (12).

### Equipment

All laboratory-scale chromatography experiments with mAb feedstock, model proteins, and model DNA were performed using an ÄKTA Explorer FPLC system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The devices were connected to the ÄKTA Explorer with standard tubing and luer-lock connectors. A flow rate of 10 membrane volume (MV)/min was used. Binding of endotoxin and bacteriophage molecules was performed using a separate experimental setup consisting of a peristaltic pump (Watson Marlow 302S), which allowed proper cleaning of the system. To determine flow rates, membrane adsorber devices were connected to a pressure vessel filled with buffer or protein solution. The filtrate volume was monitored using a balance and the flow rates for different pressures were calculated up to an inlet pressure of 3 bar.

### Model systems

Bovine serum albumin (BSA, Lot 50121326) was purchased from Kraeber GmbH & Co. and salmon sperm DNA (DNA, Lot 8087) from Biomol. The protein throughput was determined using γ-globulin (Sigma, γ–globulin from bovine blood, Lot STB0227K9). Endotoxin from *Escherishia coli* (Lonza LPS *E. coli* 055:B5 N185 Lot 0000100778) was used as standard. Bacteriophage ΦX174 (ATCC 13706-B1) was produced in a 50 L disposable bioreactor using the *E. coli* (ATCC 13706) expression system. Subsequently, phage was purified, concentrated, and sterile filtered by several steps including a depth filtration cascade, crossflow filtration, precipitation with polyethylene glycol, and centrifugation.

### MAb feedstock

The mAb feedstock was obtained from pilot-scale batches produced at Genentech (a member of the Roche Group). It was expressed in mammalian cells and clarified to remove insoluble impurities. The mAb was processed through a protein A chromatography step and

further purified using a cation-exchange chromatography step. Protein concentration was approximately 11 g/L.

## METHODS

### Dynamic binding capacity

Each device was sanitized with 1 N NaOH for 30 min at 10 MV/min followed by equilibration with 150 MV binding buffer composed of 150 mM NaCl in 20 mM Tris/HCl pH 7.3 ± 0.1, conductivity 16 mS/cm. 150 MV of 1 g/L BSA in binding buffer or 0.1 g/L DNA in binding buffer were loaded. All solutions used were prefiltered with a 0.2 µm membrane filter. All steps were performed at flow rate of 10 MV/min. Breakthrough curves were recorded by measuring the extinction at 280 nm (protein) and 260 nm (DNA) using the ÄKTA Explorer. To compare different devices the void volume of the experimental setup was determined by injection of acetone (2 %). The dynamic binding capacity at 10% breakthrough was calculated as shown in **Equation 1**,

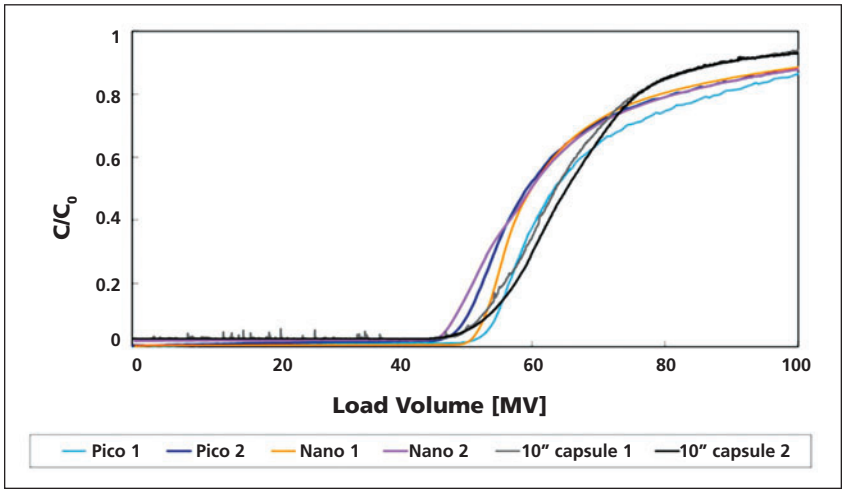
$$DBC = \frac{(V_{10\%} - V_V)^* C_i}{V_M} \quad \text{[Eq. 1]}$$

where $V_{10\%}$ is volume loaded at 10% breakthrough, $V_v$ is void volume, $V_m$ is membrane volume, and $c_i$ is initial concentration.

### Protein throughput

Each membrane adsorber device was sanitized with 1 N NaOH for 30 min at 10 MV/min followed by equilibration with 100 MV binding buffer composed of 150 mM NaCl in 20 mM Tris/HCl pH 7.3 ± 0.1, conductivity 16 mS/cm. Protein throughput was determined using the pressure vessel filled with a solution of 20 g/L γ-globulin in binding buffer. The filtrate volume up to 1000 MV was monitored at a constant pressure of 3 bar using a balance.

**Figure 5:** Bovine serum albumin breakthrough curves for pico, nano, and 10" devices. MV is membrane volume.




### Chinese hamster ovary proteins clearance

Chinese hamster ovary proteins (CHOP) clearance was determined using industrially relevant mAb feedstock. Before loading the MAb feedstock onto the membrane adsorber, the membrane was equilibrated with 10 MV of 50 mM Tris buffer at the appropriate pH. The conductivity of this buffer was adjusted by altering the concentration of sodium acetate. After equilibration, the mAb feedstock was loaded onto the devices to a targeted load density of 10 kg mAb/L of membrane at a flow rate of 10 MV/min. Pool fractions were collected during the experiment and analyzed for CHOP concentration.

### Determination of log reduction value of bacteriophages

Equipment and membrane devices were sanitized with 1 M sodium hydroxide for 30 minutes. Membrane devices were further equilibrated with 300 MV of binding buffer. The ΦX174 phage solution with a titer of $1.5x10^7$ PFU/mL was prepared and loaded onto the devices at a flow rate of 10 MV/min. Flow-through fractions were collected after 100 and 150 MV of load for quantitative analysis.

### Endotoxin removal

Pump, tubing, and devices were treated with 1 M sodium hydroxide for 30 minutes at room temperature and at a flow rate of 10 MV/min before performing the experiment. Compatible vessels and materials were heated at 200 °C for 4 hours to destroy naturally occurring endotoxins. After sufficient rinsing with reverse osmosis water, the equilibration was performed with 300 MV of binding buffer. 150 MV of endotoxin in binding buffer were loaded to the membrane at a flow rate of 10 MV/min. The flow-through was divided into fractions of 50 MV each and was analyzed to determine the endotoxin level.

## ASSAYS

### CHOP quantification

An ELISA was used for CHOP quantification. Samples containing CHOP were incubated

**Table II:** Dynamic binding capacity (DBC) at 10% breakthrough using bovine serum albumin (BSA) and DNA model molecules. BSA is bovine serum albumin.

| Device | Membrane volume (mL) | 10% DBC BSA (g/L) | 10 % DBC DNA (g/L) |
|---|---|---|---|
| Pico 1 | 0.08 | 55.83 | 9.06 |
| Pico 2 | 0.08 | 50.78 | 9.43 |
| Pico 3 | 0.08 | 50.78 | 8.94 |
| Pico 4 | 0.08 | 48.25 | 9.06 |
| **Average (Pico)** | | **51.41** | **9.12** |
| Nano 1 | 1 | 53.54 | 8.94 |
| Nano 2 | 1 | 49.11 | 9.78 |
| **Average (Nano)** | | **51.32** | **8.52** |
| 10" | 180 | 51.84 | 8.02 |
| 10" | 180 | 52.89 | 7.51 |
| **Average (10")** | | **52.42** | **7.70** |

**Table III:** Log reduction value of bacteriophage φX174 with Sartobind pico and nano devices. MV is membrane volume.

| Load volume (MV) | Pico 1 | Pico 2 | Pico 3 | Pico 4 | Nano 1 | Nano 2 |
|---|---|---|---|---|---|---|
| 100 | 5.4 | 5.1 | 5.1 | 5.3 | 5.2 | 5.5 |
| 150 | 5.5 | 4.9 | 4.8 | 5.1 | 5.3 | 5.3 |
| **Average** | 5.4 | 5.0 | 5.0 | 5.2 | 5.3 | 5.4 |

in the wells, followed by incubation with anti-CHOP antibodies conjugated with horseradish peroxidase (HRP). The HRP enzymatic activity was detected with *o*-phenylenediamine, and the CHOP was quantified by reading absorbance at 490 nm in a microtiter plate reader. Based on the principles of sandwich ELISA, the concentration of peroxidase corresponded to the CHOP concentration. The assay range for the ELISA was typically 10–320 ng/mL, with intra-assay variability of approximately 10%. CHOP values were reported in units of ng/mL. CHOP values could be divided by the mAb concentration and the results reported in units of PPM (parts per million; ng of CHOP/mg of mAb).

### Bacteriophage ΦX174 quantification

Host organism *E. coli* was used for the detection of infectious ΦX174 phage particles. *E. coli* cells were incubated on agar plates (Soybean-Casein Digest Agar Medium–Trypticase Soy Broth 211043), which served as a base layer with nutrients. *E. coli* cells multiplied rapidly and formed a bacterial lawn. Phage particles infect the cells, causing the lysis of *E. coli* host cells and producing single circular, nonturbid areas called plaques in the bacterial lawn. Each plaque represents the lysis of a phage-infected bacterial culture and is designated as a plaque-forming unit (pfu), and used to quantitate the number of infective phage particles in the culture. Plaques must be clearly defined and samples were then diluted several times (1:10) depending on the phage concentration. During the study, 150 µL of the host cell solution (optical density 2–6) was mixed with 150 µL of sample and top agar (1.3% Tryptikase Soy Agar BD 211043) and the mixture was then distributed to agar plates (4% Tryptikase Soy Agar BD 211043 in 90 mm petri dishes) and incubated for 18 to 24 hours at 37 °C. Plaque forming units were counted and the titer of the sample in PFU/mL (plaque forming units per mL) was calculated using **Equation 2**,

$$Titer = \frac{P}{E} \cdot \frac{D}{V_{Sample}} \qquad \text{[Eq. 2]}$$

**Table IV:** Endotoxin removal (log reduction value) at pH 7.3 in buffer containing 150 mM NaCl with Sartobind pico and nano devices. MV is membrane volume.

| Load volume (MV) | Pico 1 | Pico 2 | Pico 3 | Pico 4 | Nano 1 | Nano 2 |
|---|---|---|---|---|---|---|
| 50 | > 3.96 | > 3.96 | > 2.92 | > 3.96 | > 3.96 | > 3.96 |
| 150 | > 3.96 | > 3.96 | > 3.96 | > 3.96 | > 3.96 | > 3.96 |
| 150 | > 3.96 | > 3.96 | > 3.96 | > 3.96 | > 3.96 | > 3.96 |

where P is the number of plaques of all countable dilutions, E is the sum of emphasis, D is the lowest evaluated dilution, and VSample is the sample volume.

The LRV was calculated using **Equation 3**,

$$LRV = \log_{10}\left(\frac{C_0}{C_{FT}}\right) \quad \text{[Eq. 3]}$$

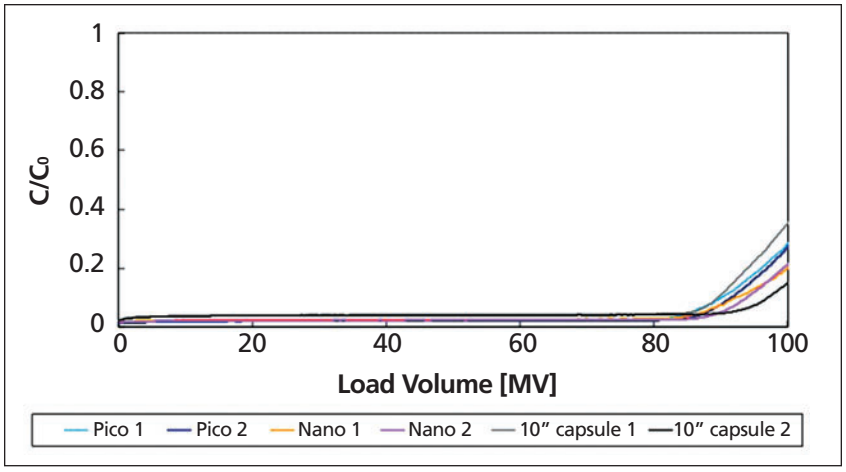
where $c_0$ was the titer of the initial solution and $c_{FT}$ the titer in the flow-through fraction.

### Endotoxin quantification

The endotoxin level was measured by the kinetic chromogenic method test according to the manufacturer's instructions (Limulus Amebocyte Lysate Chromogen, Charles River endosafe Endochrome-K R1710K, Lot A4992L 10/2012). The quantification principle is based on coloration caused by the contact of a sample containing endotoxin with a mixture of lysate and chromogenic substrate. A β-glucan blocker was added (Lonza N190 Lot 0000132199 01/11). During the 1-hour incubation the extinction coefficient was measured continuously at 405 nm using a temperature controlled (37 °C) plate reader (Tecan Safire). The reaction rate varies with endotoxin level and the samples were quantified for endotoxin by comparing the results with the calibration series. The detection limit of the assay was 0.012 EU/mL. LRV was calculated similarly to phage quantification by measuring the endotoxin level of the initial solution El0 and the level of endotoxin in the collected flowthrough fractions (ElFT) using **Equation 4**.

$$LRV = \log_{10}\left(\frac{El_0}{El_{FT}}\right) \quad \text{[Eq. 4]}$$

**Figure 6:** DNA breakthrough curves for pico, nano, and 10" devices. MV is membrane volume.



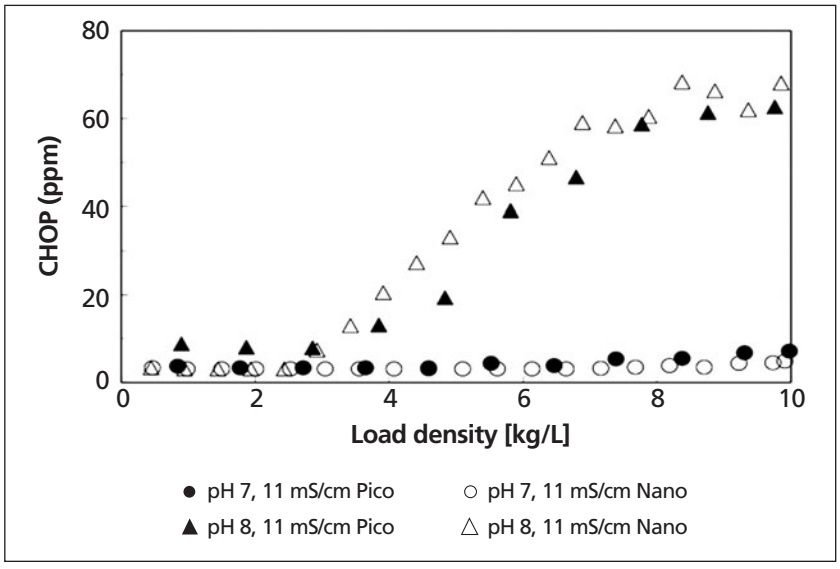

## RESULTS

### Flow rate and protein throughput

Device geometry must allow for linear scalability through the entire device size range. Pressure flow curves were generated with the axial flow Sartobind pico and radial flow Sartobind nano devices with data shown in **Figure 3**. The normalized flow rate (membrane volume (MV)/minute) increased linearly with the increasing inlet pressure and the flow rates were comparable, suggesting effective flow distribution and efficient utilization of membrane area with both pico and nano devices.

For a typical polishing application with an AEX membrane adsorber, the load capacity is very high, exceeding 10 kg of protein feedstock per liter of membrane volume and can thus present the risk of membrane fouling. To assess fouling as a function of load capacity, the Sartobind pico and Sartobind nano devices were loaded with a 20 g/L γ-globulin solution to a load capacity of 20 kg/L at a constant inlet pressure of 3 bar. As seen in **Figure 4**, while slightly higher flow decay was observed with the pico device, the overall flow decay was minimal with the two devices thus demonstrating the absence of significant membrane fouling at high load density.

**Figure 7:** Chinese hamster ovary proteins (CHOP) breakthrough curves for Sartobind pico and nano with a mAb feedstream. MAb feedstock contained 100 ppm CHOP. Experiments were performed at pH 8.0 and 7.0 at 11 mS/cm and at a flow rate of 10 MV/min.




### Characterization of membrane adsorber devices using model systems

Chromatography media are usually characterized using model molecules, with dynamic binding capacity and impurity clearance reported at specific process conditions. The dynamic binding capacity for Sartobind STIC-PA was determined using bovine serum albumin (BSA) and DNA, and impurity clearance was evaluated using DNA, endotoxin, and bacteriophage.

*Dynamic binding capacity:* The dynamic binding capacity at 10% breakthrough was measured for the Sartobind pico, the Sartobind nano, and the Sartobind SingleSep 10" capsule using BSA and DNA model systems. All devices were assembled with STIC-PA membranes. The breakthrough curves for the three devices are shown in **Figures 5** and **6** for BSA and DNA, respectively. The breakthrough curves are similar for all devices suggesting consistent flow distribution and efficient utilization of the membrane binding sites at the three scales. **Table II** shows the average BSA and DNA dynamic binding capacity values for several Sartobind pico, nano and 10" devices. At 10% breakthrough, the difference in dynamic binding capacity for all three devices was insignificant. The consistent dynamic binding capacity with BSA and DNA supports a linear scalability from 0.08 mL axial flow pico device to 180 mL radial flow SingleSep 10" capsule.

*Removal of bacteriophage:* Pathogen clearance was evaluated using the bacteriophage ΦX174, serving as a surrogate for mouse minute virus (MMV), which is typically used as a model virus for virus validation studies. Both ΦX174 (26-33 nm diameter) and MMV (20 nm diameter) are small nonenveloped DNA viruses with an isoelectric point of around 6.7–7.0 and 6.2 respectively (13). At pH > 7, both ΦX174 and MMV are mainly negatively charged and expected to bind to positively charged AEX chromatography membranes, resulting in their clearance from protein feedstock through electrostatic interactions. To compare clearance between Sartobind pico and Sartobind nano, the same ratio of ΦX174 to membrane volume was loaded. Process-scale capsules were not tested because of the large amount of phage material required. Two flow-through fractions were collected with each pico and nano device, and the LRV was evaluated by comparing the phage titers of the fractions with the load solution. As shown in **Table III**, similar LRVs were obtained at a load of 100 and 150 MV of phage-spiked buffer, demonstrating linear scalability between the devices.

*Removal of endotoxin:* Endotoxins are lipopolysaccharides found in the outer membrane of various gram negative bacteria, can be present as different forms of micelles and vesicles, and are generally strongly negatively charged. Because of their ability to elicit immunogenic responses in humans, endotoxins must be removed to typically < 0.25 Endotoxin Units per milliliter (EU/mL) where EU is the unit of measurement for endotoxin activity (*USP* <29>). **Table IV** shows the results for endotoxin removal with Sartobind pico and nano devices at pH 7.3 in a buffer containing 150 mM sodium chloride. The concentration of endotoxin in the load was 108 EU/mL, which is significantly higher than the concentration of endotoxin typically found in any in-process pools. Three fractions were collected from the flow-through at loading volumes of 50, 100, and 150 MV. All flow-through fractions had an endotoxin concentration below the detection limit of

0.012 EU/mL resulting in a LRV > 3.96 except one fraction at 50 MV with the pico device. However, subsequent fractions at higher load volumes with the same pico device provided LRV > 3.96 which suggests that the anomalous reading at 50 MV was likely due to an assay error or sample contamination. Based on the load volumes tested, the total amount of endotoxin removal was > 1296 EU with the pico and > 16200 EU with the nano device. Significantly larger amount of endotoxin would be required in the load to saturate the membrane with the endotoxin molecules to determine and compare the breakthrough curves for both pico and nano devices.

### Performance of Sartobind pico with an industrially relevant mAb feedstream

In a mAb purification process, AEX chromatography is typically operated in a flow-through mode to bind trace levels of impurities such as DNA, putative viruses, endotoxins, and host cell proteins, while the mAb product flows through. The load capacity is indicated as the mass of product loaded per unit volume of chromatography membrane (kg mAb/L membrane) such that the purity level in the product pool is acceptable. To assess the performance with an industrially relevant feedstream, both pico and nano devices were loaded with an in-process mAb pool post Protein A and cation exchange chromatography step. Subsequently, CHOP levels were monitored in the flow-through as a function of load density. The devices were loaded to 10 kg/L load density at two different solution conditions (pH 7.0 and 8.0 at 11 mS/cm). CHOP clearance as a function of load density is shown in **Figure 7.** Comparable CHOP clearance was obtained with the pico and the nano device at both solution conditions using an industrially relevant mAb feedstock, suggesting that the Sartobind pico is scalable to the Sartobind nano device. Additionally, at pH 7.0 and 11 mS/cm, a load capacity · 10 kg/L could be achieved with pool CHOP levels < 10 ppm.

The CHOP clearance results are consistent with the earlier data where comparable BSA and DNA dynamic binding capacity was observed between the pico, nano, and process scale 10“ devices. Comparable clearance of endotoxin and the bacteriophage further demonstrated the scalability of Sartobind pico to the Sartobind nano.

## CONCLUSION

It is well documented in the literature that AEX membrane adsorbers are an attractive alternative to columns for polishing applications in a flow-through mode. Because of its hydrodynamic benefits, load capacity greater than 10 kg/L of membrane can be achieved with membrane chromatography. Such high load density necessitates a significantly large amount of protein feedstock for process development and validation, which could be cost prohibitive. To overcome this limitation and also to reduce validation cost particularly for virus spiking studies, an ultra scale-down device, Sartobind pico, having a membrane volume of 0.08 mL was developed. Using model molecules and an industrially relevant mAb feedstock, Sartobind pico was compared to the existing commercial scale-down device Sartobind nano. BSA and DNA breakthrough curves, CHOP, bacteriophage, and endotoxin clearance data demonstrate the scalability of Sartobind pico to the Sartobind nano. The new scale-down pico device will facilitate the development of flow-through polishing applications for recombinant proteins and monoclonal antibodies by reducing the sample consumption by 10-fold and providing substantial cost savings for process characterization and virus validation studies.

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Photo Courtesy of Sartorius Stedim Biotech

# Polishing Complex Therapeutic Proteins

## A New Downstream Purification Platform Using a Salt-Tolerant Membrane Adsorber

Sartobind STIC (salt-tolerant interaction chromatography, Sartorius Stedim Biotech, Goettingen, Germany), a salt-tolerant anion-exchange membrane adsorber, has demonstrated proof-of-concept in removing residual host-cell impurities from high-salt, packed-bed affinity chromatography eluate. Although the new platform process using Sartobind STIC has fewer unit operations, it produces drug substance with comparable quality attributes to current processes, thus significantly improving productivity and reducing cost of goods. The study presented herein focuses on implementing a novel membrane adsorber for optimized polishing.

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Packed-bed chromatography is the main workhorse for the downstream processing of therapeutic proteins and monoclonal antibodies (mAbs). Packed-bed columns provide good binding capacity and scalability, combined with excellent resolution. The mass transfer process in packed-bed chromatography comprises several steps, including convection, pore diffusion, and film diffusion. The rate-limiting step of this process is pore diffusion (i.e., the slow diffusion of solutes into the dead-ended pores inside the chromatography media where the majority of the binding sites are located). As a result, residence time is an important parameter for column chromatography and often becomes the limiting factor of how fast the process can be run. High back pressure is another concern when operating packed-bed columns at a high flow rate. Membrane-adsorber (MA) chromatography technology was developed to overcome this mass transfer limitation (1). By coupling functional groups onto a filter-like porous matrix, diffusion-

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based mass transfer is eliminated because the filter pores are flow-through pores and allow solutes to be transported to the binding sites via convection. With MA chromatography, only film diffusion may limit mass transfer rate and the binding capacity is generally independent of the load flow rate over a wide range (2,3). Importantly, MAs can be manufactured with extremely shallow matrix beds (e.g., bed-heights in the mm range) that have very large cross-sectional area-to-volume ratio. Thus, MAs can be operated at much shorter residence time compared with packed-bed columns (e.g., seconds vs. minutes), thereby reducing the process time and increasing throughput at a large scale (4).

It has been noted for some time that an anion-exchange MA is an attractive alternative to anion-exchange columns when operated in flow-through mode to remove low levels of impurities, such as DNA, host-cell protein (HCP), and virus (5). Flow-through polishing columns are usually sized for speed to achieve desired flow rate and process time, using only a small fraction of the binding capacity available for impurity removal. Because MAs allow for a faster flow rate, a small MA device can replace a bigger column and still provide sufficient binding capacity for impurity clearance (6–8). Today, membrane chromatography has proven to be a robust alternative to Q column chromatography for polishing in flow-through mode, and multiple case studies have demonstrated the popularity of their implementation (9–13). Single-use MAs not only reduce process time, buffer usage, and floor space, but also eliminate the column packing and cleaning validation activities required for packed-bed columns. A detailed cost analysis showed that single-use Q membranes can be cost competitive compared with a reused Q Sepharose fast-flow column in a mAb process when its process capacity is sufficiently high (7). More recent analyses also show that using a disposable MA in flow-through mode provides comparable or a lower cost of goods (CoG) than using a packed-bed column (14, 15). Overall, for a flow-through polishing step, replacing a packed-bed column with a single-use MA can provide cost savings.

As with any other ion-exchange chromatography, conductivity and pH have significant effect on the performance of anion-exchange MA as a flow-through polishing unit operation. One report stated that to ensure sufficient impurity clearance, the ideal range for Sartobind Q flow-through step in a mAb process is 3–4 mS/cm at pH 7.0–7.2 (13). Similarly, another study using Q MA from Millipore found that to achieve > 1 log removal of host cell protein, the load had to be conditioned to pH 8.0 and a conductivity of < 4.0 mS/cm (6). The low salt tolerance of these Q MAs means that a dilution step prior to loading is often required to achieve desired impurity clearance, which increases process complexity. In recent years, efforts were made, both in academia and industry, to develop new types of anion-exchange MAs that have better salt tolerance, which will enable greater process flexibility and potentially lead to wider usage of MA flow-through polishing in the downstream processes. A systematic screening study by Riordan *et al*. identified three factors that contributed to salt tolerance of anion-exchange MA: ligand net charge, ligand density, and molecular structure of the ligand (16). Interestingly, the study also found that available hydrogens on the amine-

**Figure 1:** Schematic comparison of three generations of purification processes for non-mAb recombinant therapeutic proteins at Bayer. a) Older generation purification process. b) Current platform purification process. c) Future platform purification process (i.e., STIC process). A is affinity, FR is blast freeze, IA is immunoaffinity, IE is ion exchange, MA is membrane adsorber, VI is viral inactivation, VF is viral filtration, UF is ultrafiltration, and DF is diafiltration.

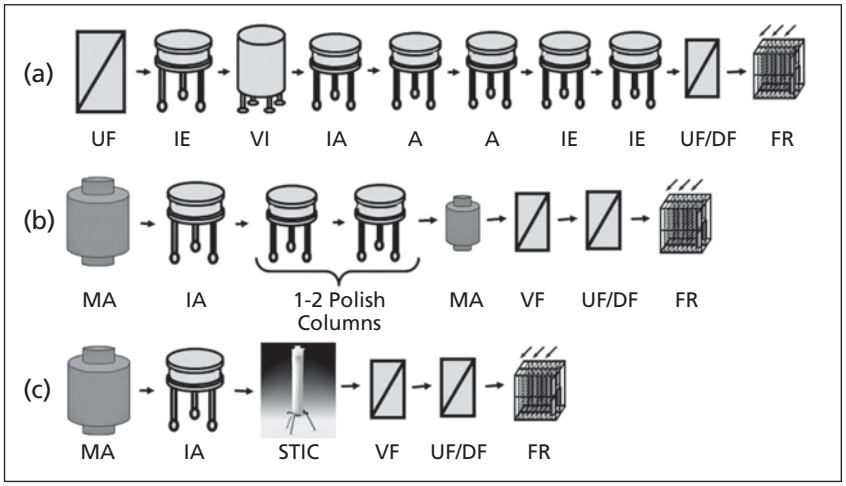


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**Figure 2:** a) Step-wise salt elution of Bay-A001 from Sartobind Q and Sartobind STIC. Brown line is conductivity trace, red line is A280 trace from Sartobind Q, and blue line is A280 trace from Sartobind STIC. Numbers at the bottom are molar concentrations of NaCl in different fractions. b) Step-wise elution of Bay-A001 from Sartobind STIC using increasing percentage of immunoaffinity elution buffer. Brown line is conductivity trace and blue line is A280 trace. Numbers at bottom are percentage points of immunoaffinity elution buffer in different fractions.

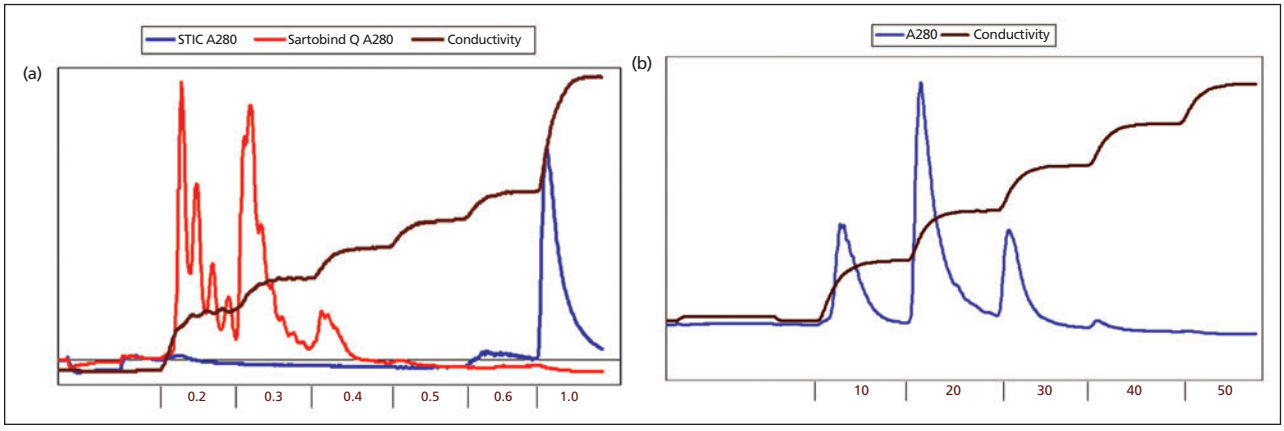



**Figure 3:** Dynamic binding capacity of host cell impurities by Sartobind STIC. a) Host cell proteins breakthrough curve with host cell protein (HCP) spike-in. b) DNA breakthrough curve with representative immunoaffinity eluate.

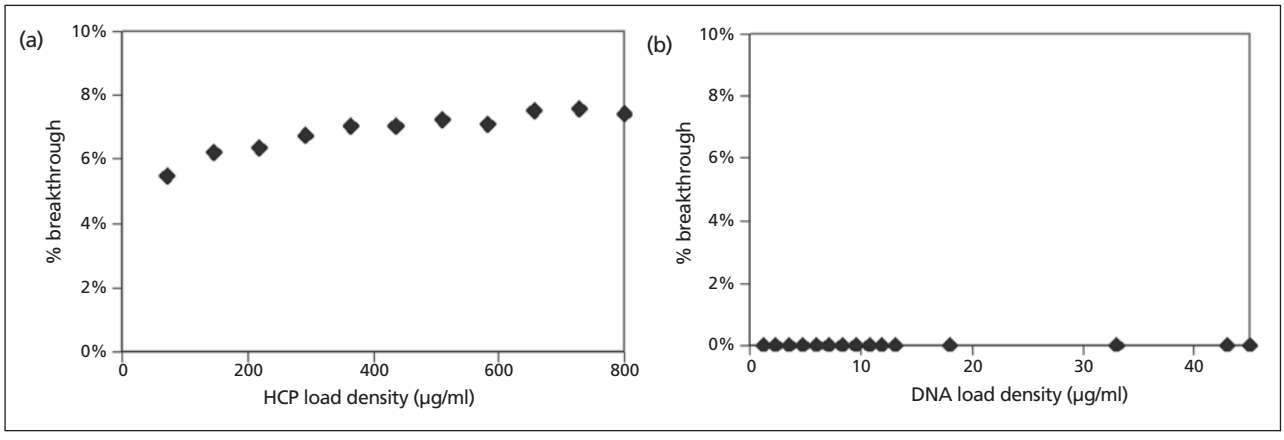



**Table I:** Process parameters of Sartobind STIC unit operation in a laboratory-scale STIC process for Bay-A001.

| Membrane bed volume | 1 mL (Nano) |
|---|---|
| Flow rate | 5 mL/min |
| pH | 7.0 @ 5 °C |
| Conductivity | 39 mS/cm @ 5 °C |
| Load density by total protein | 9 mg/mL |

binding group improved the salt tolerance of the ligand, indicating that primary amines might have better salt tolerance than quaternary amines.

Sartobind STIC (salt tolerant interaction chromatography, Sartorius Stedim Biotech, Goettingen, Germany) is a weak anion-exchange MA that is less sensitive to increasing salt concentration than standard Q membranes (17). It carries the polyallylamine ligand that provides high charge density and salt tolerance. The new double-porous membrane replaced the previous generation of membrane with hydrogel, which was shown to shrink and reduce binding site accessibility under high salt conditions (18). Sartobind STIC was shown to provide significantly higher binding capacity and higher LRV of model viruses compared with Sartobind Q in the presence of 150 mM NaCl (16.8 mS/cm) (17). This enhanced salt tolerance allows the MA polishing step to

**Table II:** Critical quality attributes of drug substances (DS) from Bay-A001 laboratory-scale STIC process are comparable to those from current process at manufacturing scale. N.D. = not detected; NA = not available. SEC–HPLC is size-exclusion chromatography–high-performance liquid chromatography, SDS-PAGE is sodium dodecyl sulfate polyacrylamide gel electrophoresis.

| | Laboratory-scale STIC Process | | | Current process average | Current process standard deviation |
|---|---|---|---|---|---|
| | Run 1 | Run 2 | Run 3 | | |
| Yield (STIC vs. 3 polishing steps) | 93% | 89% | 97% | 92% | 14% |
| DS HCP (μg/dose) | 0.4 | 0.6 | 0.7 | 0.4 | 0.1 |
| DS DNA (pg/dose) | 2.8 | N.D. | N.D. | N.D. | NA |
| DS aggregates by SEC–HPLC | 1.5% | 1.0% | 1.1% | 0.6% | 0.3% |
| DS degradation product by SEC–HPLC | 4.0% | 7.3% | 6.8% | 4.2% | 0.4% |
| DS purity by SDS–PAGE | 96% | 96% | 94% | 94% | 1% |
| DS specific activity | 4887 | 4239 | 3846 | 5483 | 184 |
| DS Mouse IgG | < 0.5 ng/mL | NA | NA | < 0.5 ng/mL | NA |

be conducted without load dilution, thus reducing process time and complexity.

This article describes the development work at Bayer to evaluate Sartobind STIC as a platform polishing unit operation for complex protein therapeutics. Specifically, the authors looked at product yield, and HCP and DNA removal by Sartobind STIC from very high salt intermediates, and how to improve the overall purification platform. Data suggest that using a salt tolerant MA enables the number of unit operations in a platform process to be reduced, potentially reducing cost of goods.

## MATERIALS AND METHODS

Sartobind Q and Sartobind STIC membrane adsorbers in LP15 (0.41 mL) and nano (1 mL) formats were provided by Sartorius Stedim (Goettingen, Germany). The ligand for Sartobind STIC is a polyallylamine compared to the quaternary amine for Sartobind Q. The LP15 prototype is a 0.41 mL disk format device with three membrane layers in a polysulfone housing. The design of the LP15 device is similar to the commercially available syringe unit Sartobind MA15. Sartobind nano is the commercially available scale-down device with 15 layers and 36.4 $cm^2$ total surface area. Scalability of Sartobind nano (1 mL) to process scale capsules has been well demonstrated through the entire range of product up to 1.62 L membrane. Cylindrical format, radial flow distribution, and bed height were kept constant to allow for linear scale up.

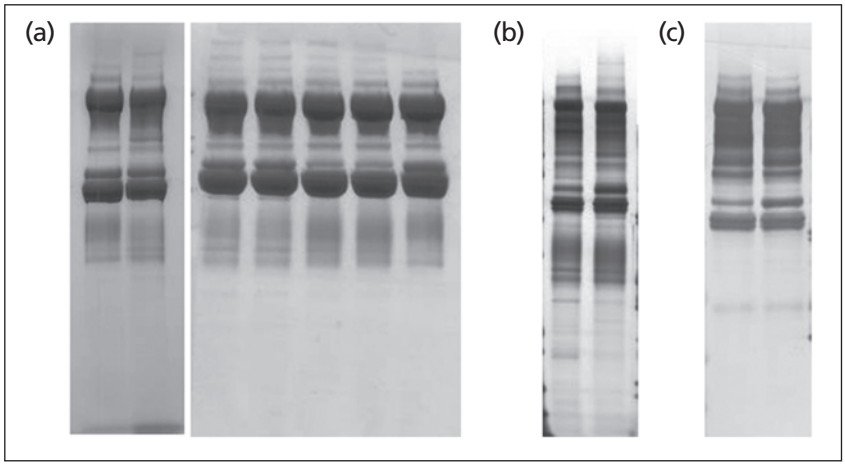
The recombinant protein feed materials for this study were obtained from clinical manufacturing at Bayer Berkeley, CA. The immunoaffinity columns used were directly scaled down from clinical manufacturing processes.

As in current processes, all purifications were carried out at 2–8 °C to obtain maximum product stability. Step gradient experiments and breakthrough experiments were carried out on an AKTA Explorer 100 chromatography system from GE HealthCare (Uppsala, Sweden). Simple flow-through runs were carried out using a Watson Marlow 101U peristaltic pump (Falmouth, England). Initial development studies used Sartobind STIC LP15 devices at a flow rate of 3 mL/min (7.3 MV/min). Sartobind STIC nano was used for laboratory scale purifications of two different complex glycoproteins, Bay-A001 and Bay-A002, at a flow rate of approximately 5 mL/min (5 MV/min). The full factorial study on pH and conductivity was designed and data analyzed in JMP (SAS, Cary, NC). All buffers used in the anion exchange MA operation were imidazole based. No multivalent anion, such as phosphate or citrate, was used as it may interfere with protein adsorption because of its strong interaction with AEX ligands.

Bay-A001 and Bay-A002 have theoretical isoelectric points (pI) of 7.4 and 6.4, respectively, based on the amino acid sequences. However, due to the heterogeneity of post-translational modifications, the recombinant proteins exhibit high levels of heterogeneity in pI, which prevented pI determination using isoelectric focusing (IEF) electrophoresis.

All assays were performed by the analytical development and support group at Bayer, Berkeley, CA.

**Figure 4:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of Bay-A001 drug substances generated from laboratory-scale STIC process. a) Commassie blue staining. From left to right: Lane 1: Bay-A001 reference standard, Lane 2: lab scale run 1, Lane 3: lab scale run 2, Lane 4: lab scale run 2, Lane 5: lab scale run 3, Lane 6: lab scale run 3, Lane 7: Bay-A001 reference standard. b) Silver staining, left lane: Bay-A001 reference standard, right lane: laboratory-scale run 1. c) Western blot, left lane: Bay-A001 reference standard, right lane: laboratory-scale run 1.




## RESULTS

### Current platform purification process for Bayer recombinant protein therapeutics

To reduce the cost of development and the time to clinic, the downstream process development team at Bayer recently established a platform purification process for Bayer's complex recombinant protein therapeutics (**see Figure 1b**). An older generation purification process is also included in **Figure 1** for comparison (**see Figure 1a**). Not only does the current process have fewer unit operations, but it also uses more modern separation technologies, resulting in significantly higher yield and shorter process times, thus reducing the cost of goods.

Two of the unit operations in the current platform process use MA technology. A Q-MA capture step operating in bind-and-elute mode was developed for quick isolation of unstable, low concentration protein products from large volumes of cell-culture harvest generated by perfusion-based bioreactors. The fast flow property of MA allowed us to quickly concentrate and stabilize the products, which will otherwise gradually lose activity in the crude harvest.

Following the MA capture step, an immunoaffinity column was used to provide the majority of purification power of the entire process. Because of the products' sensitivity to non-neutral pH, elution from the immunoaffinity column was achieved with a high concentration of chaotropic salt instead of a pH change. As a result, the eluate was of very high conductivity and a dilution step, in some cases more than 10-fold, was needed before proceeding to the next unit operation. To further remove trace amount of impurities, one or two polishing columns were included after immunoaffinity. The second Q-MA step was a flow-through step designed specifically for removal of residual DNA. To ensure good product recovery, the MA polishing step operated under fairly high salt concentration (> 20 mS/cm at 5 °C), under which no HCP or viral clearance was observed. A viral filtration step provided robust non-enveloped virus clearance and enhanced the pathogen safety profiles of the products. Lastly, a ultrafiltration/diafiltration (UF/DF) step concentrated and formulated the drug substance for frozen storage.

The salt tolerance of Sartobind STIC may provide the opportunity to further improve the process by potentially adding HCP and/or viral clearance capability from high-salt feed streams, reduce the need for dilution, and reduce the number of unit operations.

### Sartobind STIC has greater binding strength than Sartobind Q

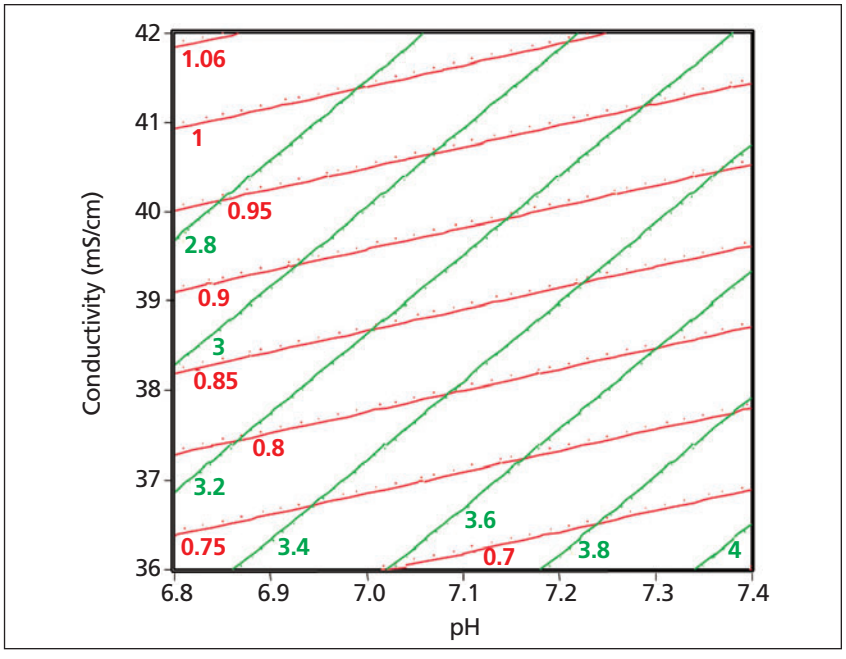
The first step was evaluating the level of salt tolerance of Sartobind STIC in comparison with Sartobind Q (see **Figure 2a**). Purified Bay-A001, a Bayer recombinant protein product, was loaded to either Sartobind Q or Sartobind STIC under neutral pH and low salt concentration so that the protein binds to the membranes. A step-wise NaCl gradient wash was conducted to determine the NaCl concentration necessary to elute Bay-A001 from each MA. Some split peaks and conductivity fluctuations were observed, which were probably caused by non-ideal flow distribution inside the LP15 membrane holder. Running the same gradient through the bypass line did not produce any conductivity fluctuation (data not shown). This flow distribution issue, however, did not affect the interpretation of the

**Table III:** Full factorial design of experiments (DOE) screening study shows the effect of pH and conductivity on yield and host-cell protein (HCP) clearance. N.D. = not detected.

| Run # | pH | Conductivity (mS/cm) | Yield | HCP fold clearance | Host cell DNA in flowthrough (pg/mL) |
|---|---|---|---|---|---|
| 1 | 7.4 | 42 | 104.8% | 2.92 | N.D. |
| 2 | 6.8 | 36 | 79.6% | 3.01 | 3 |
| 3 | 7.4 | 36 | 58.2% | 4.36 | N.D. |
| 4 | 6.8 | 42 | 99.1% | 2.84 | N.D. |

results. The elution of Bay-A001 from Sartobind Q started in the 0.2 M NaCl fraction and continued in the 0.3 and 0.4 M NaCl fractions. This product was inherently heterogeneous and the elution into multiple fractions as shown in the study was consistent with what was seen before. In comparison, no significant elution was observed from Sartobind STIC at NaCl concentrations up to 0.6 M. An elution peak was observed in the last fraction with 1 M NaCl, although the integrated peak area was significantly smaller than the total integrated peak areas from the Sartobind Q chromatogram. Because the same amount of protein was loaded to each MA, the data indicated that not all protein was eluted from Sartobind STIC at 1 M NaCl. Overall, this experiment demonstrated the salt tolerance of Sartobind STIC. It also showed that a high salt concentration was needed to ensure good product recovery for processing proteins, such as Bay-A001, in a flow-through mode using Sartobind STIC.

**Figure 5:** Contour plot of pH and conductivity on Sartobind STIC yield and host-cell protein (HCP) clearance. Red lines and numbers represent predicted yield, and green lines and numbers represent predicted HCP fold clearance. The data used to generate this contour plot are listed in Table III. DNA levels in Sartobind STIC FT are either below or close to the limit of detection of 2.5 pg/mL, so no contour plot was generated for DNA clearance.

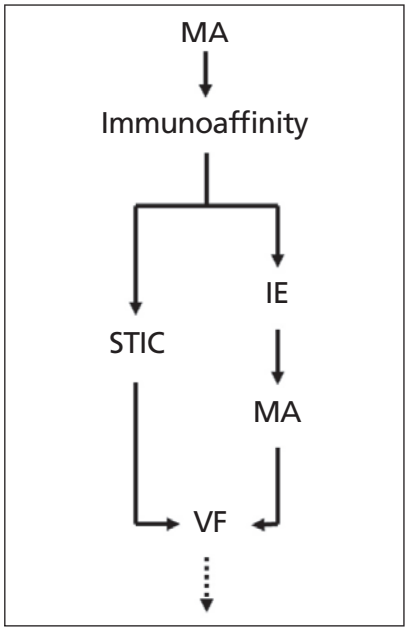



### Sartobind STIC as a polishing step for high-salt immunoaffinity eluate

The possible implementation of Sartobind STIC into the platform purification process was evaluated. One option was to use Sartobind STIC to replace the current Q MA polishing step, which served solely as a DNA removal step without the requirement for any feed stream adjustment. The drawback, however, was that a salt addition into the feed stream would be needed to ensure good recovery from Sartobind STIC. This added operation contradicted the goal of process improvement. The only step in the process where the salt concentration was high enough for Sartobind STIC flow-through operation was the immunoaffinity eluate. It was therefore decided to use Sartobind STIC to polish the immunoaffinity eluate in flow-through mode to remove residual DNA and HCP. The salt tolerance feature of Sartobind STIC meant that less dilution of the immunoaffinity eluate was needed. The impurity clearance performance by Sartobind STIC would decide whether any other polishing steps were required.

An important parameter for the proposed Sartobind STIC operation was the maximum dilution on the immunoaffin-

**Figure 6:** Sartobind STIC was tested to replace two unit operations in Bay-A002 purification process. The step yield is 96%. The host cell protein (HCP) in STIC FT is 0.1 µg/dose, a 6-fold reduction from STIC load and comparable to the HCP level in current process. The DNA level in STIC FT is below the limit of detection.




ity eluate, which should give a salt condition high enough to give good product recovery, while maximizing impurity removal. To determine the dilution target, purified Bay-A001 was loaded to a Sartobind STIC LP15 device at low salt concentration. The membrane was then washed with a 0–50% stepwise gradient of the immunoaffinity elution buffer (see **Figure 2b**). The chromatogram showed that with 30% immunoaffinity elution buffer, almost all the proteins were eluted. The conductivity of the 30% immuno-affinity elution buffer solution was measured to be 39 mS/cm at 5 °C.

The authors then investigated whether Sartobind STIC could clear DNA and HCP under the same buffer conditions. HCP spike-in for HCP clearance evaluation was chosen because the low HCP level in the immunoaffinity eluate and its high salt content prevented the authors from getting reliable assay results. A small aliquot of immunoaffinity load material, which had HCP as the major protein content, was spiked into a diluted immunoaffinity elution buffer at 39 mS/cm at 5 °C. The solution was loaded to a Sartobind STIC LP 15 device and flow-through fractions were collected. Interestingly, the HCP assay showed an immediate 5–7% breakthrough (see **Figure 3a**), which remained steady at HCP load densities up to 800 µg/mL membrane. Because HCP consisted of a mix of proteins, it was likely that some of the more basic proteins did not bind to Sartobind STIC under the testing conditions, causing immediate breakthrough. Nevertheless, HCP clearance observed in this experiment was significant and possibly sufficient to reduce HCP to a level within the acceptable range for the Bay-A001 drug substance. For the evaluation of DNA clearance, Bay-A001 immunoaffinity eluate was diluted to 39 mS/cm at 5 °C and loaded to a Sartobind STIC LP15 device. Flow-through fractions were collected and the DNA content in load and flow-through fractions was analyzed by quantitative polymerase chain reaction (qPCR). As shown in **Figure 3b**, no DNA can be detected in the flow-through at DNA load densities up to 45 µg/mL membrane volume. The DNA load density was limited by the availability of feed material and the maximum DNA binding capacity was expected to be much higher. A DNA binding capacity of 24 g/L for Sartobind STIC at 16.7 mS/cm was previously reported (17). Overall, it appeared that by diluting Bay-A001 immunoaffinity eluate to 39 mS/cm at 5 °C, Sartobind STIC in flow-through mode could be used to remove DNA and HCP with good product recovery. Although the maximum impurity load densities achieved in these studies were not high, they still represented good process throughput because the impurity concentrations in immunoaffinity eluate were very low.

With its DNA and HCP clearance capability from immunoaffinity eluate, Sartobind STIC has the potential to replace polishing columns and the Q MA polishing step in the platform process. For the new platform process, which was tentatively called STIC process, there are only three chromatography unit operations: a Q MA capturing step, an immunoaffinity column, and a Sartobind STIC flow-through polishing step (see **Figure 1c**). To determine whether STIC process could produce Bay-A001 drug substance comparable to the current platform process, three laboratory-scale purification trains using STIC process were performed. Viral filtration was not performed in the laboratory-scale runs. Based on previous experience, viral filtration can reduce HCP and aggregates in Bay-A001 by approximately two-fold. The process parameters for the laboratory-scale Sartobind STIC step are listed in **Table I**. **Table II** compares the performance of laboratory-scale STIC process with that of the current platform process at manufacturing scale. Sartobind STIC has an average step yield of 93%. The combined yield of the three polishing steps that Sartobind

STIC replaced is 92%. Thus, no difference in overall yield was expected between the two processes.

Critical quality attributes of the drug substances from the two processes were also compared. HCP levels from STIC process are low and comparable to those from the current process, even without the additional clearance from viral filtration. Two STIC process runs had DS DNA levels below detection, as is the case with the current process. The other STIC process run had DS DNA at 2.8 pg/dose, significantly lower than the specification for this product and regulatory guidelines. Aggregates were about two-fold higher in DS from STIC process compared with the current process. However, aggregate levels were expected to be comparable if viral filtration is included in the STIC process based on our experience that the viral filter provides about two-fold reduction in aggregates. Degradation product levels tested slightly higher in STIC run 2 and 3, but were well within acceptable range and should not be a quality concern. Purities as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Coomassie blue staining were comparable between the two processes. Specific activity was somewhat lower with STIC process, but still within the acceptable range. Data analysis showed that the lower specific activities were correlated to lower step yields at the laboratory scale UF/DF and not directly caused by STIC, which could be an equipment-specific issue. More extensive comparability studies at a larger purification scale following this proof-of-concept will show whether this difference is consistently obtained. The immunoaffinity column does not have significant ligand leaching based on previous experience. Thus, the IgG clearance capability of Sartobind STIC was not characterized. For both processes, the levels of mouse IgG in drug substance were below the assay's limit of detection. **Figure 4** shows the SDS-PAGE analysis, including Coomassie blue staining, silver staining, and Western blotting, of STIC process drug substance in comparison with Bay-A001 reference standard. No unknown band or significant change in band pattern was observed in STIC process drug substance. Overall, the STIC process reduced the number of unit operation by two compared with the current platform process, without sacrificing yield or product quality.

## pH and conductivity are critical parameters for Sartobind STIC operation

To evaluate how pH and conductivity variations may affect Sartobind STIC performance, a full factorial screening DOE study was designed in JMP with a pH range of 6.8–7.4, and a conductivity range of 36–42 mS/cm at 5 °C. Aliquots from Bay-A001 immunoaffinity eluate were adjusted to pH and conductivity targets, as listed in **Table III**. Each aliquot was then loaded to a Sartobind STIC LP15 device. Step yield, DNA, and HCP clearances from each run were also listed in **Table III**. A contour plot showing trends of yield and HCP clearance in response to pH and conductivity changes was generated in JMP (see **Figure 5**). As expected from any anion-exchange flow-through operation, increasing pH decreased product yield but increased HCP clearance, while increasing conductivity increased product yield but decreased HCP clearance. Robust DNA clearance was observed because DNA levels in STIC FT from all four runs were either at or below the limit of detection. Careful control of pH and conductivity is, therefore, crucial for ensuring robust performance of Sartobind STIC.

## The performance of Sartobind STIC with Bay-A002

To demonstrate that Sartobind STIC can be a platform unit operation, performance in processing another Bayer recombinant protein product, Bay-A002, was tested. As outlined in the platform purification process (see **Figure 1b**), Bay-A002 is also captured from cell-culture harvest using a large-scale Q MA in bind-and-elute mode, followed by purification using an immunoaffinity column. An ion-exchange column and a Q MA flow-through step were used to further polish the product. The process was tested using Sartobind STIC in flow-through mode to polish the high salt immunoaffinity eluate, which was diluted to a conductivity of 39 mS/cm at 5 °C for STIC loading. A laboratory-scale purification run showed that Sartobind STIC gave an excellent yield of 96%. It reduced HCP to 0.1 µg/dose, a six-fold reduction, and reduced DNA to below the limit of detection. These HCP and DNA levels were comparable to those in the MA flow-through in the current process, indicating that Sartobind STIC can replace both the ion-exchange column and the Q MA steps (see **Figure 6**). Because anion-exchange is a versatile purification technique, the

authors believe Sartobind STIC can be easily adapted to processing various proteins by finding the optimal pH and conductivity settings for each protein, thus making it a true platform technology.

## DISCUSSION

In this study, proof-of-concept for using Sartobind STIC as a platform-polishing unit operation was achieved. When operated in flow-through mode, Sartobind STIC is capable of removing HCP and DNA from high-salt feed streams with good product recovery. A new purification process incorporating Sartobind STIC has fewer unit operations than the current platform process, but produces drug substance with similar yield and comparable quality attributes. Sartobind STIC allows a further streamlined, future manufacturing platform for complex recombinant proteins. This new platform will have only three chromatography unit operations: a reusable Q MA capturing step, an affinity column providing the majority of purification, and a single-use Sartobind STIC polishing step. This one-column-two-MA platform process is well suited for purifying low titer, unstable, complex proteins, which are an important part of Bayer's biologics pipeline. With fewer unit operations and a single-use polishing step, the new platform process is expected to reduce process time, increase productivity, and reduce the cost of goods.

Q MA in flow-through mode also provides viral clearance in mAb processes (19). The conductivity for the process, however, has to be low (3–4 mS/cm) to prevent viral particles from breaking through the membrane. Low conductivity is often feasible with mAbs because many mAbs have high pI and will not bind to an anion-exchanger, even at low conductivity. XMuLV and PPV clearance by Sartobind STIC was tested under the process conditions described in this paper. No significant clearance was observed at 30–39 mS/cm at 5 °C. Because of the strong binding of the products to Sartobind STIC, the conductivity cannot be lowered further to achieve viral clearance without severely affecting product yield. The lack of viral clearance, however, does not disqualify Sartobind STIC as a platform polishing step. For example, no viral clearance claim was made on any of the polishing steps in the Bay-A001 process; hence, replacing those polishing steps with Sartobind STIC as shown in **Figure 1** has no effect on the viral clearance claims of the process. With proteins that bind less strongly to anion-exchangers, such as mAbs, Sartobind STIC could provide significant viral clearance at conductivity settings higher than what would be required for a Q MA step. This could be a significant advantage that may eliminate the need for a dilution step.

The authors will continue to evaluate Sartobind STIC for processing new protein therapeutics, including mAbs, in Bayer's pipeline and will also seek opportunities to test the new platform process at the pilot scale to demonstrate its scalability. It could also be beneficial to evaluate other salt tolerant anion exchange MAs, such as ChromaSorb from EMD Millipore, for the same application. These new-generation membrane adsorbers show how new ligand chemistry and new matrix structure can lead to improved separation performance, which is not achievable with older generation chromatography media. This type of technological innovation allows continuous improvement of platform manufacturing processes, as demonstrated in this study.

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# Development of an Alternative Monoclonal Antibody Polishing Step

In many monoclonal antibody (mAb) purification platforms, traditional anion exchange column chromatography or, increasingly, anion exchange membrane chromatography, is used as a polishing step in a product flowthrough mode to bind trace levels of process- or product-related impurities and assure efficient viral clearance. Anion exchange chromatography is, however, limited by the requirement for low loading buffer conductivity to efficiently remove impurities, which necessitates buffer exchange or dilution of the protein A column eluate. In this study, the authors developed a mAb polishing step using salt tolerant interaction membrane chromatography. Using a 96-well high-throughput screening (HTS) approach the authors identified the initial chromatographic parameters for acceptable step recovery and product quality. The authors then confirmed these conditions using small STIC capsules. Using a combination of HTS screening and design of experiments optimization the authors developed a mAb polishing platform which demonstrated high step recovery and efficient clearance of impurities (i.e., host cell proteins, high molecular weight species, host DNA, and leached protein A) for multiple antibodies at higher loading buffer conductivity. This simple and efficient polishing step can be easily integrated into most current mAb purification platforms, which may shorten mAb purification processes and accelerate development programs.

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KRIS PERSAUD, DALE LUDWIG, AND PAUL BALDERES

Monoclonal antibody (mAb) purification processes exist in different well-established platforms with extensive process performance histories for production of commercial monoclonal antibodies (1–10). These platforms, typically employing two or three chromatographic steps, are scalable and robust, and produce proteins with acceptable process yield and product quality.

In most of the two-column downstream processing platforms, the first chromatographic unit operation is protein A which binds the target mAb product directly from the harvested cell culture fluid (3, 4, 10–12). The process impurities are removed in the flowthrough and subsequent wash steps. A low pH buffer elutes the product and sets up the subsequent viral inactivation step. Anion exchange chromatography (AEX), such as Q Sepharose Fast Flow (Q FF) column chromatography (3, 13, 14) and Q membrane adsorber (6, 15–18), serves as the second chromatographic purification step. It is operated in a flowthrough mode, binding trace impurities such as host cell proteins, host DNA, endotoxins, and in some instances, high molecular weight (HMW) species while the antibody passes through. The AEX chromatography step is limited by the requirement for low loading buffer conductivity, which necessitates buffer exchange through tangential flow filtration (TFF) or dilution of the protein A column elution pool for efficient impurity clearance. However, some antibodies may have solubility issues at low ionic strength conditions. These challenges may be addressed by Sartorius Sartobind salt tolerant interaction chromatography (STIC) using a polyallylamine ligand covalently coupled to the double-porous membrane (19). The optimized base support membrane matrix combined with weak anion exchange chemistry provides a robust method for viral clearance at physiological conductivities and above (19, 20). A virus, ΦX174, used to model weak acidic contaminants, was shown to be removed (LRV $> 5$) in the presence of 150 mM NaCl. Megta et al. demonstrated efficient viral clearance on STIC using two model viruses, MMV and MuLV (21). Furthermore, similarly to Q membrane chromatography, the STIC membrane adsorber may also provide some economic benefits as an alternative mAb polishing step (16, 22).

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In this study, Sartobind STIC was evaluated as a mAb polishing platform alternative to Q column chromatography or Q membrane adsorber. Using a combination of high-throughput screening (HTS) and design of experiments (DOE) optimization, we developed a STIC mAb polishing platform which demonstrated high step recovery and efficient clearance of impurities (host cell proteins, host DNA, and leached protein A) for four antibodies at higher loading buffer conductivity. In addition, since there is no need for buffer exchange, the pre-Q column TFF step can be removed from the purification process. This polishing step, which can be easily integrated into current mAb purification platforms, offers a viable alternative to traditional AEX especially in cases where antibodies exhibit poor process performance. Furthermore, methods described here for developing STIC operating conditions can be applied to the purification process development of other membrane adsorbers.

## MATERIALS AND METHODS

### Purification techniques

The mAbs used for this study were fully human IgG1 produced in recombinant Chinese hamster ovary (CHO) cells grown in a serum-free medium. MabSelect SuRe protein A (GE Healthcare, Piscataway, NJ) was used to purify the antibody present in the harvested cell culture fluid (HCCF) using AKTAexplorer under the control of UNICORN 5.0 (GE Healthcare, Piscataway, NJ). Briefly, the protein A column was loaded to approximately 35 mg mAb/mL-resin. The product was eluted using 50 mM acetate buffer, pH 3.5–3.8, which was mapped out for each protein on 96-well plates. Necessary wash steps were introduced to reduce the host cell protein (HCP) level in the eluate. The protein A elution pool was held at room temperature for one hour after pH was adjusted to 3.5 using 1 M acetic acid for viral inactivation. Following low-pH treatment, the product pool was neutralized to the required pH with 2 M Tris base solution, clarified through a 0.22 μm filter (EMD Millipore, Billerica, MA), which served as the feed to STIC experiments.

**Table I:** Four model antibodies partially purified by MabSelect SuRe protein A. HMW is high molecular weight, HCP is host cell protein.

| Antibody | pI | HMW (%) | HCP (ppm) |
|---|---|---|---|
| Mab-D | 7.6–8.0 | 0.5–3 | 125 |
| Mab-K | 9.2–9.4 | 0.5–2 | 85 |
| Mab-S | 7.8–8.4 | 0.5–2 | 200 |
| Mab-T | 8.9–9.4 | 2–5 | 300–600 |

The STIC equilibration buffer conditions were first screened using 96-well plates (gifts of Sartorius Stedim Biotech, Bohemia, NY) with a full factorial design of experiments. The buffer condition was evaluated at 5 pH levels of 6.5, 7.0, 7.5, 8.0, and 8.5, and 6 NaCl concentrations of 0, 25, 50, 75, 100, and 150 mM. Before loading into each well of STIC plates, the protein A eluate was adjusted to the appropriate pH using 2 M Tris base solution and to the target salt concentration using 5 M NaCl stock solution. The flowthrough/subsequent wash from each well was collected as the product. Response parameters, process yield, HCP, and HMW were determined for each experimental run. The response surfaces were defined in a group of optimization experiments using a 96-well STIC plate through a central composite design with 4 center points. All experimental design and data processing were performed using JMP version 8.0 software (SAS Institute, Cary, NC).

Antibody dynamic loading capacity (DLC) on Sartobind STIC was determined at the optimized buffer pH and NaCl conditions using 1 mL STIC Nano capsule (Sartorius Stedim Biotech, NY) at 10 membrane volume (MV)/min. We collected different flowthrough fractions and determined the HCP level in each fraction. The DLC value was the antibody amount applied to the membrane adsorber when HCP in the flowthrough reached 10 ppm or 20 ppm when applicable. The bound materials in the case of Mab-T were eluted using 50 mM Tris, pH 7.2, 2.0 M NaCl and analyzed for the level of HMW species. The process and product related impurities in the STIC purified products were determined using different analytical techniques.

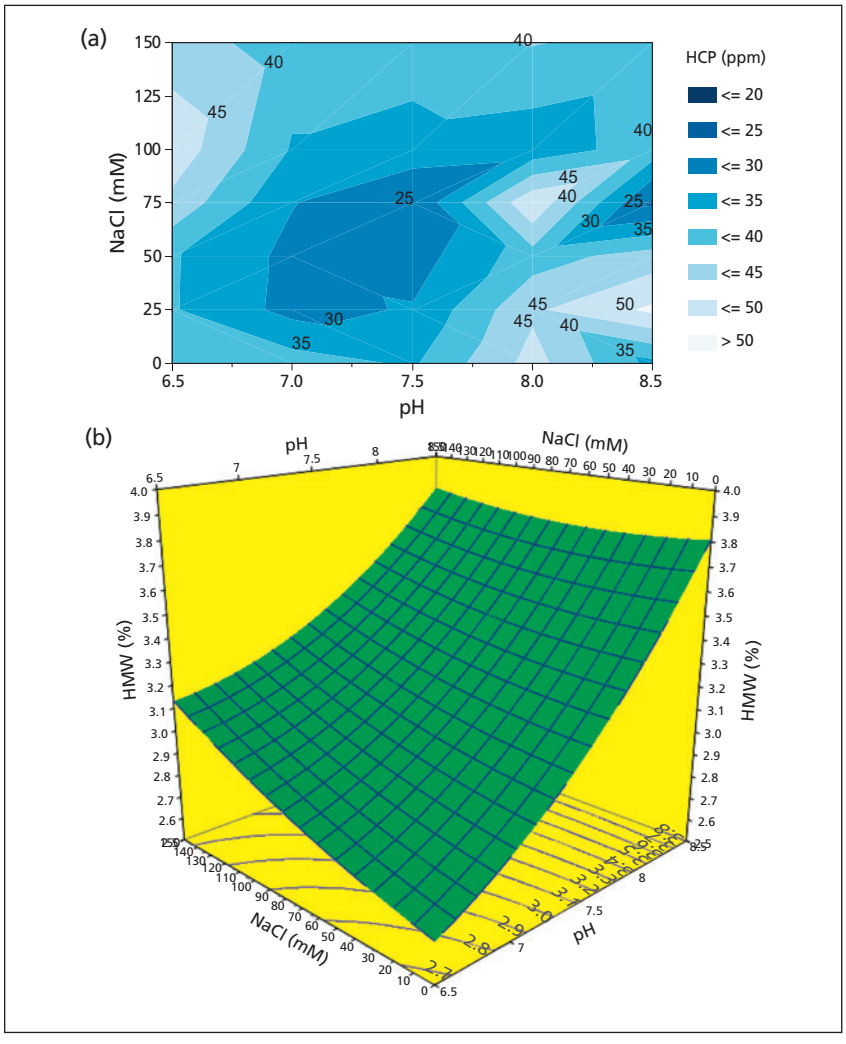
### Analytical techniques

Antibody concentrations in purified solutions were determined by the absorbance at 280 nm, using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Size exclusion high performance liquid chromatography (SE–HPLC) was used to monitor the size heterogeneity of mAbs under native conditions on Agilent HPLC system using ChemStation as the controlling software (Santa Clara, CA). A TSK-Gel G3000SW$_{XL}$ column (Tosoh Bioscience, Montgomeryville, PA) was utilized to separate HMW species, monomers, and fragments. The mobile phase was phosphate buffer saline (without $Ca^{2+}$ and

**Table II:** Dynamic loading capacity of antibodies on Sartobind salt tolerant interaction chromatography (STIC) Nano at 10 ppm HCP breakthrough.

| Antibody | Capacity (kg/L-STIC) |
|---|---|
| Mab-D | 3.5 |
| Mab-K | 3.7 |
| Mab-S | 3.0 |
| Mab-T | 0.5 |

**Figure 1:** Operating condition screening of Mab-T purification on Sartobind salt tolerant interaction chromatography (STIC) using a full factorial design. HCP is host cell protein, HMW is high molecular weight species.




Mg2+), pH 7.2 (Life Technologies, Carlsbad, CA).

A CHO host cell protein (CHOP) kit (Cygnus Technologies, Southport, NC) was used to determine the residual HCP level in purification in-process samples and purified mAb product during the screening stage of experiments according to the manufacturer's protocol. The HCP level in antibodies purified on STIC Nano was also measured by electrochemiluminescence (ECL) technology (Meso Scale Discovery or MSD, Gaithersburg, MD) developed at ImClone. Briefly, 25 µL of 3 µg/mL in-house purified anti-CHOP capturing antibodies were immobilized overnight on a 96-well MSD plate. The plate was blocked for 1 h with 3% BSA at room temperature. 25 µL of mAbs in 2-fold serial dilutions and HCP standards were added into the plate and incubated for 2 h at room temperature. The bound HCPs were detected by addition of 25 µL of biotinylated anti-CHOP probe at 3 µg/mL, which was then detected by the addition of 25 µL of streptavidin conjugated sulfo-Tag at 3 µg/mL. After the completion of reaction, 150 µL of MSD buffer was added and the plate was read with MSD SECTOR Imager 2400 for relative electrochemiluminescence units (ECLU). The intensity of the ECLU was proportional to the amount of residual HCP present in antibodies by extrapolation from the standard curve with a quantification limit of 16 ng/mL. All HCP results were normalized to the in-house CHOP standards.

The leached MabSelect SuRe ligand in antibodies was determined using the RepliGen's protein A ELISA kit (Waltham, MA) with a detection limit of 0.1 ng/mL according to the manufacturer's protocol. Residual CHO DNA in antibodies was measured by quantitative PCR (qPCR) using the resDNASEQ quantitative CHO Kit (Life Technologies, Carlsbad, CA), combining high-recovery PrepSEQ sample preparation and TaqMan based-quantitation. The assay was developed at ImClone using in-house CHO DNA standards. The quantification limit of the assay was 0.1 pg/mL.

ALL FIGURES ARE COURTESY OF THE AUTHORS

## RESULTS AND DISCUSSION

### Condition screening and optimization using 96-well plates

Using protein A column chromatography under our platform operating conditions, we first prepared four antibodies, which served as model proteins to evaluate STIC as an alternative antibody polishing platform to AEX chromatography. These partially purified proteins and their properties are shown in **Table I**. Among them, Mab-D and Mab-S showed poor solubility at low ionic strength solution conditions (< 5 mS/cm), which posed challenges to our current purification platform process. Mab-T was considered as the worst-case scenario material in terms of levels of residual HCP and HMW impurities. Thus it was used here to illustrate the procedure of condition screening and optimization. Process yield, HCP, and HMW were evaluated during the condition screening and optimization. Although the study described here focused on HCP and HMW, a similar method could be applied for other impurities.

The STIC equilibration buffer conditions were first screened using a Sartorius Sartobind STIC 96-well plate in a full factorial experimental design, as described in the Materials and Methods section. The load eluate (or flowthrough) and wash from each well, representing the purified product from an experimental run, were collected and evaluated for yield, HCP, and HMW. More than 90% process yield was achieved in all 30 experimental runs. The residual HCP and HMW levels in the STIC purified Mab-T were summarized in **Figure 1**. The residual HCP was <50 ppm at all tested conditions. Higher HCP removal was achieved when the operating conditions moved to the center of pH-NaCl contour plot (see **Figure 1a**). In most cases, for a given NaCl concentration, with increasing pH, HCP removal efficiency increased to the highest point and then started to decrease. This finding suggests that the optimal pH operating window for Mab-T is at pH 7.0–7.5.

**Figure 2:** Operating condition optimization of Mab-T purification on Sartobind salt tolerant interaction chromatography (STIC) using a central composite design.

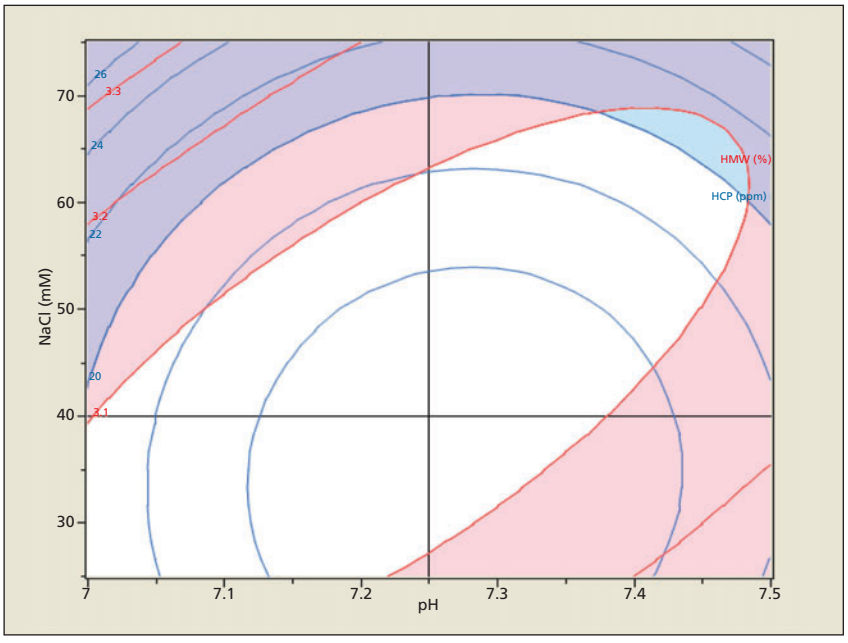
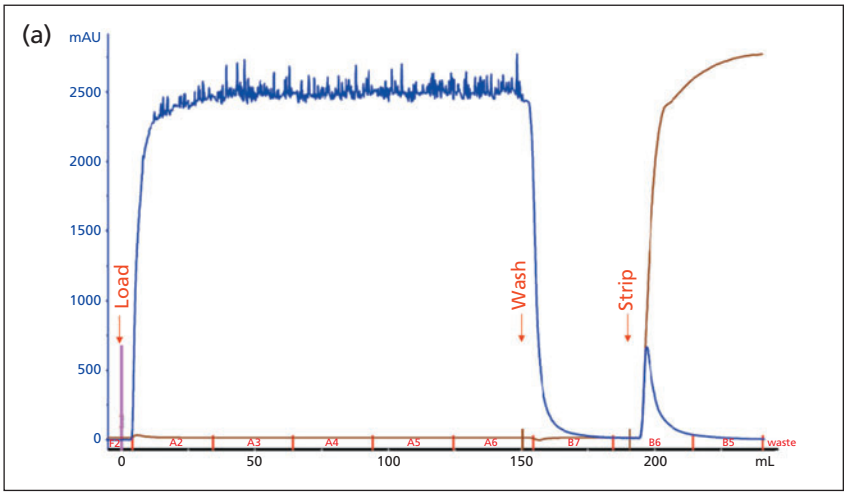



**Figure 3a:** Chromatogram of Mab-T mAb purification using Sartobind salt tolerant interaction chromatography (STIC) Nano. Buffer condition: pH 7.25, 8.33 mS/cm; Mab-T mAb concentration: 6.9 mg/mL; Impurities in the starting materials: high molecular weight species: 2.0%, host cell proteins: 372 ppm; Flow rate: 10 mL/min.




The presence of an optimal operating pH window is consistent with the amine protonation hypothesis reported previously (21, 23). As pH increases from 6.5 to 8.5, amine groups are less protonated. Thus, positive charges on the ligands available to bind impuri-

**Figure 3b:** Determination of dynamic loading capacity of Mab-T mAb on Sartobind salt tolerant interaction chromatography (STIC) Nano in terms of residual host cell protien (HCP) level.

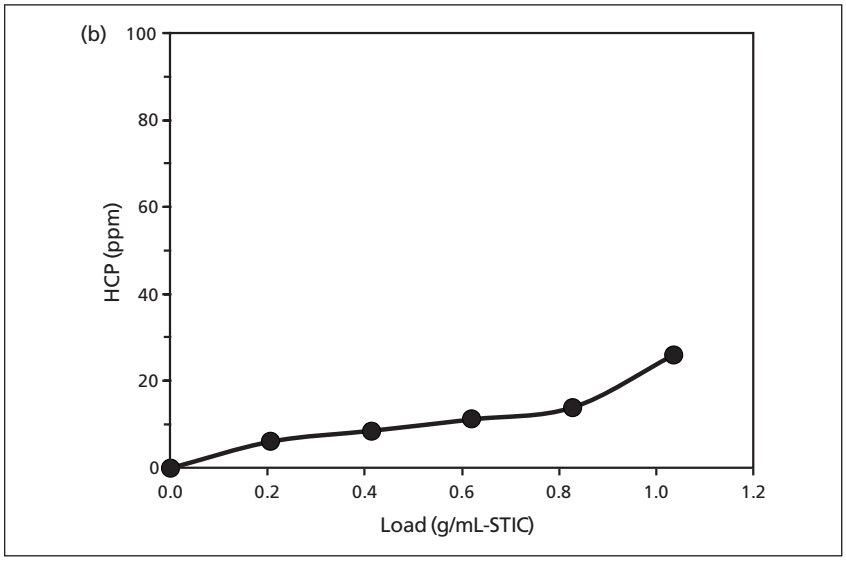
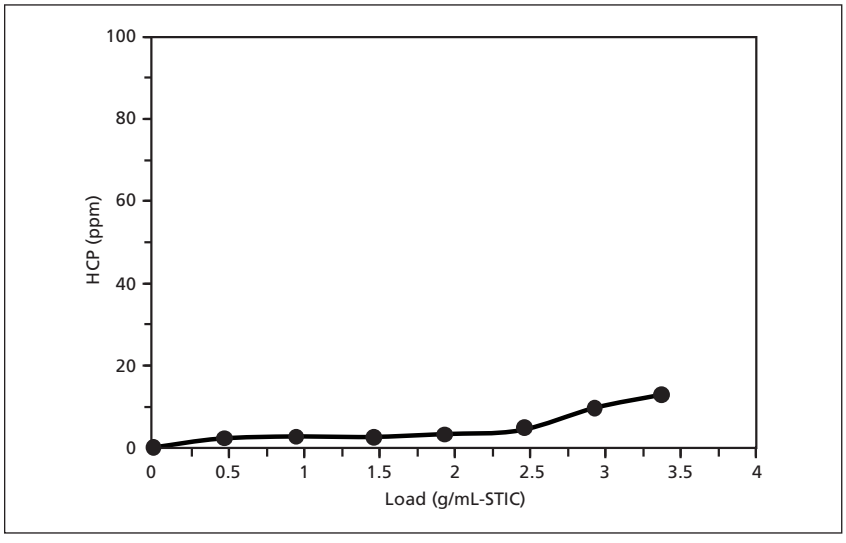



**Figure 4:** Determination of dynamic loading capacity of Mab-S monoclonal antibody on Sartobind salt tolerant interaction chromatography (STIC) in terms of residual host cell protein (HCP) level. Buffer condition: pH 7.0, 9.53 mS/cm; Mab-S mAb concentration: 12.6 mg/mL; Impurities in the starting materials: high molecular weight (HMW) species: 0.9%, HCP: 212 ppm; flow rate: 10 mL/min.




ties decrease (21). Meanwhile, with increasing pH, there is an increase in the net negative charge of host cell proteins, which results in more efficient binding to the positively charged ligands on the membrane adsorber. The presence of an optimal pH operating window is due to the combination of amine protonation on the STIC membrane adsorber and changes in protein surface charges.

In addition, in the pH range of 6.5–8.0, HCP removal was not dramatically affected by NaCl concentration, supporting the salt tolerant nature of the STIC membrane adsorber. Through this quick, full factorial DOE study using 96-well plates, optimal buffer conditions for HCP removal were identified.

We next examined the impact of equilibration buffer conditions on the removal of HMW from the partially purified Mab-T. With the understanding that in most cases, HMW level can be controlled to below 2.0% through pre-polishing steps, the goal of HMW removal in this study was to reduce HMW species from 5.0% in the load to 3.0% in the flowthrough. When operating pH was increased from 6.5 to 8.5, the HMW in the purified Mab-T increased from 2.7% to 4.0% as shown in **Figure 1b**. A concomitant decrease in the IgG monomer was observed, suggesting that the HMW removal was less efficient as the pH increased. By contrast, HMW removal was not sensitive to NaCl concentration, particularly in the range of 20–120 mM NaCl. These findings further suggest that the process performance of Sartobind STIC is a result of its salt tolerant nature, supporting a wide design space of solution ionic strength or NaCl concentration. In order to reduce the HMW in the final product to 3.0% and HCP to less than 30 ppm, pH 7.0–7.5 and 25–75 mM NaCl were selected for further condition optimization.

The initial buffer conditions developed in the screening experiments were further optimized through 12 additional experimental runs on a STIC 96-well plate via a central composite design (pH: 7.0–7.5, and NaCl concentration: 25–75 mM). The STIC response surfaces of process yield, residual HCP, and HMW level, were defined based on these runs. Again, each well in a STIC 96-well plate represented one unique combination

of experimental conditions. As expected, > 94% process yield was achieved in all experimental runs. The sweet spot of the equilibration buffer conditions is illustrated as a pH-NaCl contour plot (see **Figure 2**). When STIC was operated in the window of pH 7.2–7.3, and 30–60 mM NaCl, HCP was reduced to a lower level (< 20 ppm) and HMW to 3.0%.

### Dynamic loading capacity using STIC Nano

Breakthrough curves were used to determine the DLC of antibodies on 1 mL STIC Nano membrane adsorber. When the low-pH-treated protein A eluate of Mab-T was neutralized to the pH value (defined in the previous Condition Screening and Optimization section) and clarified, the conductivity fell into the optimal operating window. This conditioned protein A eluate was then directly applied into the STIC Nano in flowthrough mode. The Mab-T chromatogram is shown in **Figure 3a**. As expected, the sharp rising shape of the breakthrough curve during the load and sharp decreasing UV trace in the wash step suggest that mass transfer in STIC membrane adsorber is convective flow, and not limited by diffusion as in the case of porous chromatography resins. This finding is consistent with the results of previous works on other membrane adsorbers (15–17, 24). In addition, compared with Q column chromatography, the product pool was not diluted significantly by the wash, as the load volume was 150 MV while the wash volume was only 5 MV (see **Figure 3a**). Thus, STIC might provide the benefit of a lower dilution factor because of the smaller volume of buffer required in the wash step, which is extremely valuable when

**Figure 5:** Process performance analysis in terms of process yield and impurity clearance. HMW is high molecular weight species, CHOP is Chinese hamster ovary host cell protein, ProA is protein A.

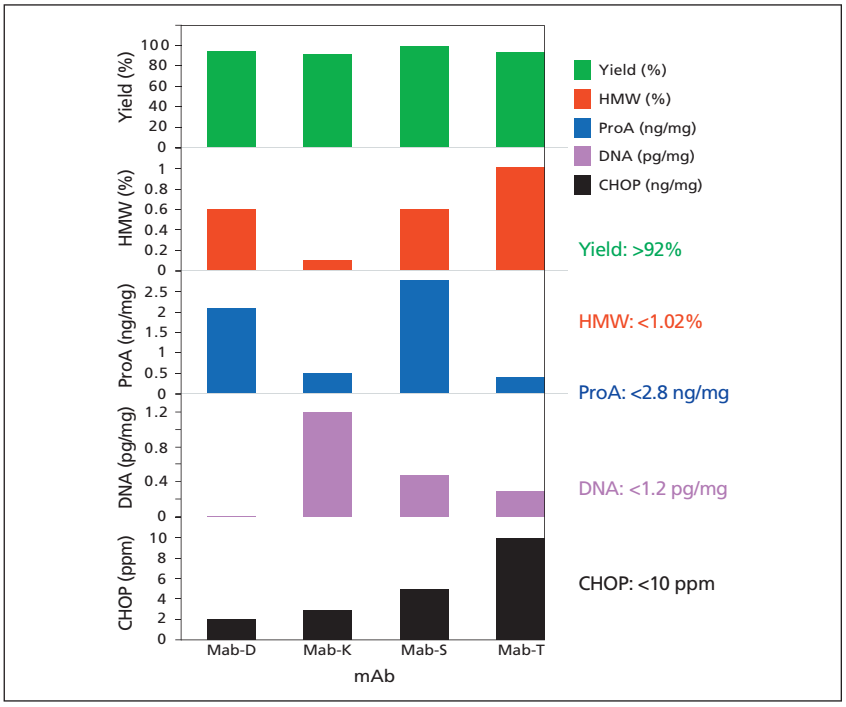



**Figure 6:** Comparison of host cell protein (HCP) removal (in fold reduction value) in two Sartobind salt tolerant interaction chromatography (STIC) formats: 96-well plates vs. 1-mL Nano capsules. 0.3 g Mab-T/mL-STIC was loaded in all of the experiments under the same operating conditions (pH 7.25, 50 mM NaCl), and the flowthrough was collected as the product. The data were analyzed using one-way Anova and the difference between plates (n=5) and capsules (n=4) was evaluated using Student's t-test.

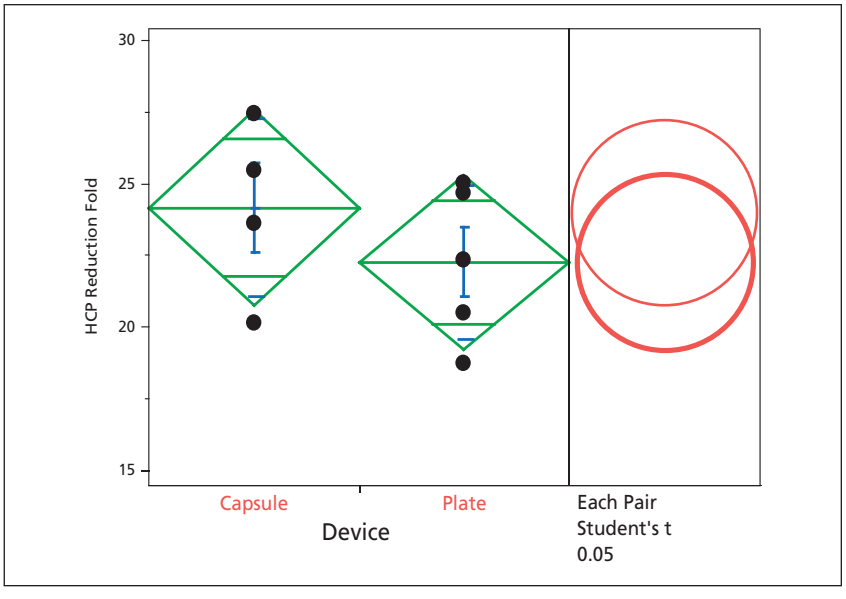
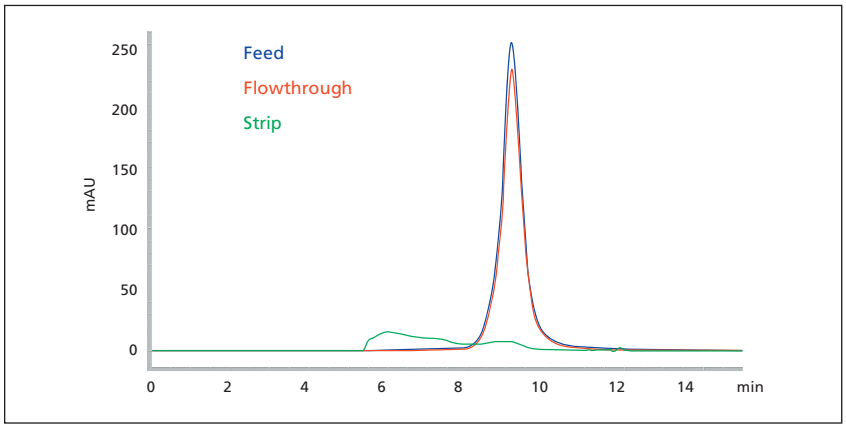


**Table III:** Comparison of traditional anion exchange (AEX) column chromatography and salt tolerant interaction chromatography (STIC) membrane adsorber.

| | Traditional AEX Column Chromatography | STIC Membrane Adsorber |
|---|---|---|
| Resin or membrane volume for processing 50 kg mAb (flowthrough mode) | > 200 L | 5 L |
| Capital equipment investment | High | Low |
| Footprint | Large | Small |
| Operations | Packing/unpacking/cleaning/storage, cleaning validation required | Disposal |
| Non-value added time | Long | Short |
| Labor requirement | High | Low |
| Consumables | High | Low |
| Buffer tanks | Large | Small |
| Development requirement | Major development effort | Plug and play |
| Mass transfer | Pore diffusion | Convective flow |
| Flow rate | Low | High |
| Salt tolerant | No | Yes |
| Process capacity per L | Low | High |

**Figure 7:** Size exclusion high performance liquid chromatography assay of high molecular weight species removal from Mab-T on Sartobind salt tolerant interaction chromatography (STIC) Nano.




there is a limit on tank capacity in manufacturing.

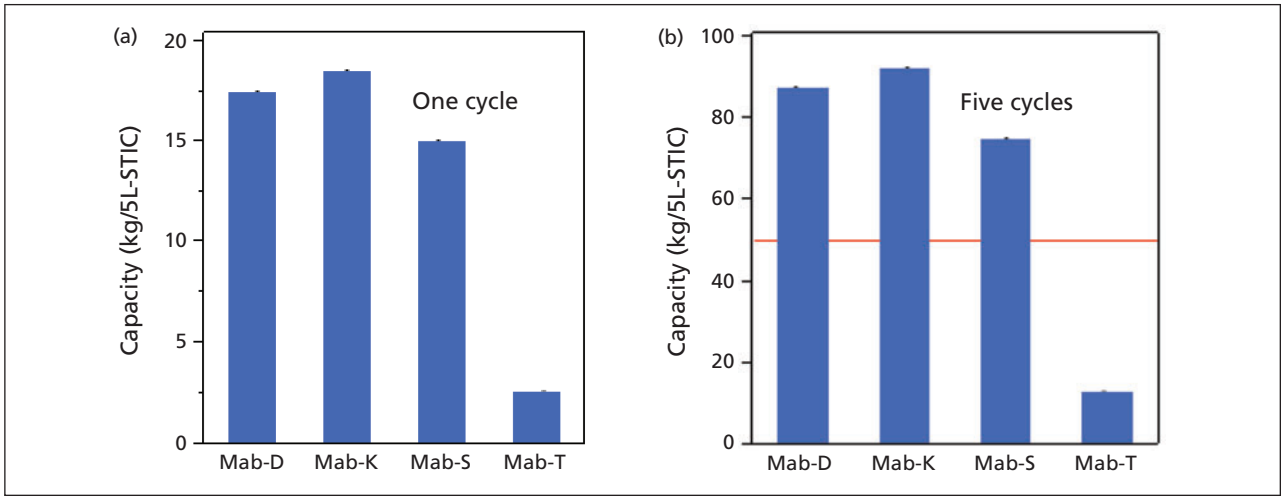
We examined the HCP breakthrough by collecting different flowthrough fractions and determining the HCP level in each fraction. As shown in **Figure 3b**, Mab-T dynamic loading capacity was 0.5 g Mab-T/mL-STIC at 10 ppm HCP breakthrough or 0.9 g Mab-T/mL-STIC at 20 ppm breakthrough, which was higher than that achieved from Q column chromatography in a flowthrough mode (data not shown).

The same screening and optimization procedures using 96-well plates were also applied to three other antibodies, Mab-S, Mab-D and Mab-K. The DLC was determined under the conditions defined by screening and optimization experiments. For Mab-S, an equilibration buffer at pH 7.0 and 9.53 mS/cm, equivalent to 60 mM NaCl, was used in the experiment. Again, different flowthrough fractions were collected and HCP was determined. With increasing load of Mab-S, HCP in the flowthrough remained at a background level up to 2.5 g mAb/mL-STIC (see **Figure 4**). After that HCP started to increase gradually and reached 10 ppm at 3.0 g mAb/mL-STIC. By contrast, only 0.5 g /mL-STIC DLC was observed for Mab-T. The significant difference in the capacity might be due to the initial HCP level (578 ppm for Mab-T vs. 212 ppm for Mab-S), as well as the initial HMW level (2.0% for Mab-T vs. 0.9% for Mab-S). Similarly, the DLC for Mab-D and Mab-K was 3.5 and 3.7 g-mAb/mL-STIC, respectively (see **Table II**), under the tailored operating conditions developed on 96-well plates. Mab-D and Mab-K thus showed a reasonably high process capacity on the STIC membrane adsorber.

## Process performance analysis

After the low-pH-treated protein A eluate of Mab-T was neutralized to pH 7.25 and clarified, it

**Figure 8:** Large-scale production scenario using salt tolerant interaction chromatography (STIC) as an alternative polishing step. Harvested cell culture fluid (HCCF): 11,000 L at a titer of 5 g/L; the target host cell protein (HCP) level is 10 ppm in the purified drug substance. a. Production capacity using 5-L STIC capsule in one cycle. b. Production capacity using 5-L STIC capsule in five cycles.




was directly applied into 1 mL STIC Nano at a loading capacity of 0.5 g/mL-STIC in flowthrough mode. As expected, 94% of Mab-T was successfully recovered in the flowthrough/wash pool (see **Figure 5**). The residual HCP was reduced to 10 ppm while aggregates were reduced to 1.02%. Furthermore, levels of residual DNA and leached protein A in the STIC purified mAb-T met the requirement for drug substance.

We also examined whether residual impurities were efficiently removed through the STIC membrane adsorber for Mab-D, Mab-K and Mab-S (see **Figure 5**). The residual HCP was reduced to less than 10 ppm. The clearance of residual DNA and leached protein A were sufficient to meet product specifications. The STIC thus served as a polishing step for Mab-D, Mab-K and Mab-S with acceptable process performance and product quality.

## Salt tolerant nature of STIC membrane adsorber

In traditional ion exchange chromatography, the interaction strength of proteins with resin ligands is controlled by solution pH and NaCl concentration. At a given pH, the interaction decreases dramatically with increasing NaCl concentration. Thus, both product recovery and impurity clearance are functions of pH and NaCl concentration, which were evaluated as the critical operating parameters during the condition screening and optimization in this study. As shown in **Figure 1**, at a given pH condition, HCP clearance did not vary significantly with NaCl concentration, which highlighted the unique salt tolerant nature of the STIC membrane adsorber. A consistent process performance has been achieved in the tested conditions of pH and NaCl concentration (see **Figures 2 and 5**), indicating a wide design space for the STIC polishing step.

It should be noted that both Mab-D and Mab-S have solubility issues in the current Q equilibration buffer (conductivity $< 5$ mS/cm), which posed challenges when the current purification production process and related operating conditions were applied. A low process yield was observed at both pre-Q column TFF and Q column polishing steps. When the solution ionic strength was increased to overcome the solubility issue, the separation efficiency of the Q column chromatography diminished appreciably. In both cases, STIC provided a viable alternative to the Q polishing step, able to remove trace amount of impurities from antibodies at a higher ionic strength buffer condition. This strategy can be applied to other antibodies, exhibiting similar solubility issues as Mab-D and Mab-S.

## Transition from 96-well plates to capsules

There are three layers of STIC membrane in 96-well format compared with 15 layers in the Nano capsule, which has the same number as the large-scale STIC devices. An equivalent fold of impurity removal was achieved using Nano capsules compared to 96-well plates with the same process load under identical operating conditions as shown in **Figure 6**. This might be due to the fact

that interactions between antibodies and STIC ligands vary with solution conditions, not with the number of layers of membrane or membrane volume. Parameters collected on the 96-well plate can thus be applied to Nano capsule. However, as reported previously, the antibody loading capacity (or process capacity) is dependent on both solution conditions and membrane volume or number of layers (24). The DLC should be determined from a small STIC membrane capsule such as Nano or Pico, which can be directly applied to larger membrane adsorbers.

### Removal of HMW species using STIC membrane adsorber

All four model mAbs were derived from stable CHO cell lines and partially purified using MabSelect SuRe resin under current platform operating conditions. It was reasonable to believe that the host cell protein profiles in these protein A-purified materials were similar. The highest HCP load was, 578 ppm, or 0.578 mg in 1 g of antibody (in the case of Mab-T). In addition, residual DNA and leached protein A only accounted for a small portion of STIC binding capacity (data not shown). The STIC binding sites could not be saturated by residual impurities at a process load of 1 g antibody/mL-STIC. The mechanism behind the lower HCP clearance during Mab-T STIC purification was therefore investigated.

Since HMW species may, through multiple-site attachment, have greater avidity to the AEX resin or membrane adsorber than the monomers, AEX in a flowthrough mode was used for HMW removal as previously reported (12, 17). Practically, the removal efficiency through AEX in a flowthrough mode varies with different antibodies. For some antibodies, HMW can be reduced to a very low level while in other cases HMW removal is not efficient, and in some extreme cases, HMW reduction is not observed at all. Thus HMW removal is challenging and should be evaluated for each case. Additionally, HMW removal with AEX resins may be limited by steric hindrance (25), indicating a potential issue with respect to loading capacity. In the case of membrane chromatography, the mechanism of mass transfer is convective flow. Therefore, the HMW binding capacity on STIC is expected to be much higher.

**Figure 7** shows HMW removal from partially purified Mab-T with 1.49% HMW in the load, assessed by SE–HPLC. The HMW in flowthrough was 0.99% with a load of 2.0 g Mab-T/mL-STIC. The amount of HMW bound to STIC in this experiment was 10 mg. However, as expected, HCP removal efficiency decreased slightly as the residual HCP in the flowthrough was 40 ppm. In this case, the HMW species might have stronger interactions with STIC than HCPs. The saturation of binding sites on the membrane adsorber by the HMW species prevented further removal of trace impurities. However, for the three other antibodies tested, either the HMW level in the load was low or only minimal HMW removal was observed, and a much higher process capacity was achieved (based on HCP breakthrough). Thus, caution should be taken if HMW species at an elevated level (> 5%) are applied to the membrane chromatography. A competitive binding analysis of HMW species and other trace impurities should be performed. If STIC offers the same or higher clearance of HMW compared to other impurities, process capacity might be compromised. Depending on the scale of purification production, different strategies can be used to mitigate the issue. Membrane chromatography in flowthrough mode with different mechanisms such as hydrophobic interaction can be incorporated. In addition, multiple cycles of STIC operation can be used to provide enough manufacturing capacity.

### Prediction on large-scale purification production

**Figure 8** presents a mAb purification production scenario using STIC as an alternative polishing step at large scale. In this theoretical case, the starting materials are proteins partially purified using a protein A column from 11,000-L HCCFs at a titer of 5 g/L. The antibody load for STIC is 50 kg. Four cycles of 5-L STIC membrane adsorber operation can provide enough production capacity for Mab-D, Mab-K and Mab-S. Unfortunately, application of STIC to Mab-T in large scale is predicted to be challenging due to its lower process capacity or higher residual HCP level. Incorporation of a HMW mitigation step in the process would be required before being applied to STIC.

The major characteristics of STIC membrane adsorbers are further compared with traditional AEX columns in **Table III**. A smaller membrane adsorber device can provide required production capacity, and reduce the plant footprint. As a single-use system, the STIC membrane adsorber avoids issues experienced in the packing, unpacking, cleaning, and storage of traditional chromatographic columns. Significant

amounts of consumables (e.g., water for injection, buffers, cleaning solutions) are saved and, more importantly, less related labor is required when membrane chromatography is used. In contrast to the major development effort that AEX chromatography requires, process development for STIC membrane adsorber is simple and efficient as demonstrated in the previous sections. Furthermore, the integrity of the membrane adsorber can be assessed using a pre- and post-use filter integrity test protocol, which is straightforward compared to the HETP test used in traditional chromatographic columns. Lastly, because of its unique hydrodynamic characteristics, membrane adsorbers can operate at a much shorter residence time or higher operating flow rate than columns, thus reducing overall processing time and costs. Therefore economic benefits can be achieved using membrane adsorbers for manufacturing of antibodies as described previously (22).

In summary, STIC provides an alternative to the current AEX polishing step. The two-column production platform can be shortened by removing the pre-AEX TFF or dilution step. It is extremely valuable for antibodies which have solubility issues at low ionic strength conditions. In addition, fast screening and optimization followed by process capacity determination in this article suggests an extremely short development timeline. More importantly STIC can be incorporated into our current platform in a "plug and play" development approach.

## CONCLUSION

The application of the STIC membrane adsorber enables an alternative polishing platform for monoclonal antibodies. An operating window of STIC in a flowthrough mode has been established through the use of high throughput screening and optimization on 96-well plates in a relatively short time frame. Acceptable product recovery and efficient clearance of host cell proteins, leached protein A, DNA, and high molecular weight species have been demonstrated on STIC Nano using four model proteins. The possible implication of HMW removal through STIC has been addressed. This simple and efficient polishing step can be integrated into current mAb production platforms.

## ACKNOWLEDGMENTS

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Photo courtesy of Sartorius Stedim Biotech

# Virus Filtration Using a High-Throughput Parvovirus-Retentive Membrane

## The authors describe testing and validation processes for Virosart HF, a surface modified polyethersulfone hollow-fiber parvovirus filter.

SUSANNE ROEDERSTEIN AND VOLKMAR THOM

All biotechnology products derived from animal sources carry a risk of contamination with viruses, including those endogenous to the source material, such as retroviruses (1) and those introduced adventitiously during manufacturing by personnel or contaminated raw materials. Viruses in biopharmaceutical products could potentially be transmitted to patients with dire consequences, particularly if the patient is immunocompromised (2). However, no such events have been reported in the context of recombinant proteins produced by fermentation because of the rigorous safety standards applied during manufacturing, including dedicated steps for virus removal and/or inactivation and a program of tests to ensure these steps are efficient, based on the guidelines set out in ICH Q5A (2).

Current regulatory guidelines require at least two orthogonal steps for the inactivation and/or removal of viruses, thus different principles of separation/inactivation must be used in each method (2,3). Because viruses vary in size, charge, and the presence or absence of an envelope, the available methods dif-

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fer in their effectiveness against particular types of virus; these factors must be integrated into the design space during the development of a process ahead of Phase I clinical trials. Manufacturers are also expected to deliver a virus clearance strategy that has been optimized for each product because biopharmaceuticals are often large, complex proteins that resemble smaller viruses in their physical and chemical properties. Virus clearance strategies must, therefore, be tailored to avoid product loss. The virus removal steps must then be tested against at least two model viruses representing those most likely to be present in the process stream (2–3) and should include an endogenous virus if relevant to the process. Ideally, an adventitious virus such as minute virus of mice (MVM) or porcine parvovirus (PPV) should also be tested, because these are the gold standards for size-dependent clearance steps using 20-nm filters (2–4). Removal/inactivation is usually demonstrated in spiking studies, where specific viruses are added deliberately to the process stream ahead of the relevant unit operations. Before Phase III clinical trials can be authorized, two further viruses must be tested if specific contaminants are likely in the process stream. The two testing schemes have different aims: general virus clearance is designed to test process robustness, whereas specific virus clearance using anticipated contaminants aims to ensure product safety (4).

Although virus clearance should be built into the design space on a product-by-product basis, several robust and effective strategies have become established in the industry (5–6). Appropriate methods for virus inactivation include heating/pasteurization or solvent/detergent treatments (7), although these may also have a significant negative impact on some recombinant proteins and are only effective against enveloped viruses. More often, a low-pH hold (8) is used for enveloped viruses if this is compatible with the buffer conditions in the process (e.g., during the production of monoclonal antibodies); exposure to ultraviolet light in the UVC range is used to inactivate all viruses by cross-linking the nucleic acids at 254 nm (9). Virus removal methods physically separate virus particles from the feed stream, and the most suitable methods are chromatography, where virus particles are captured by adsorption (10), and retentive filtration using 20-nm filters, which eliminates even the smallest viruses by size exclusion (2–4). However, there are currently no common standards for virus filtration. Instead, it is left to manufacturers to show,

**Figure 1:** (a) Hollow fiber cross section. (b) Comparison of a 0.8/2.4-m² process module and a 5-cm² laboratory module with vent filter for contained flushing.

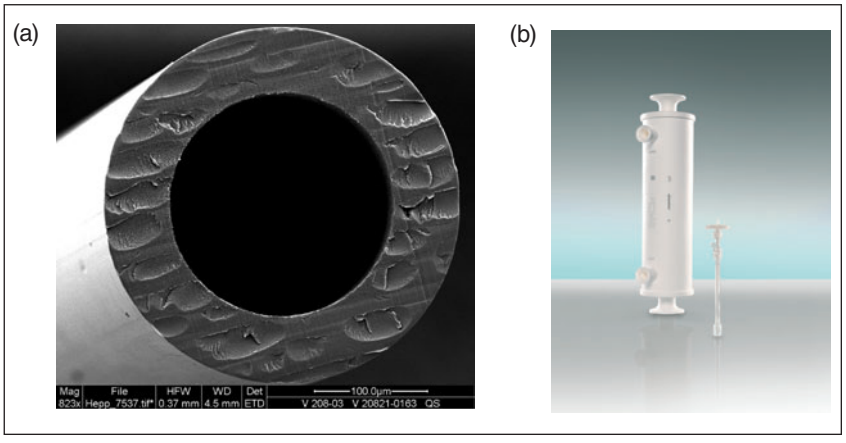
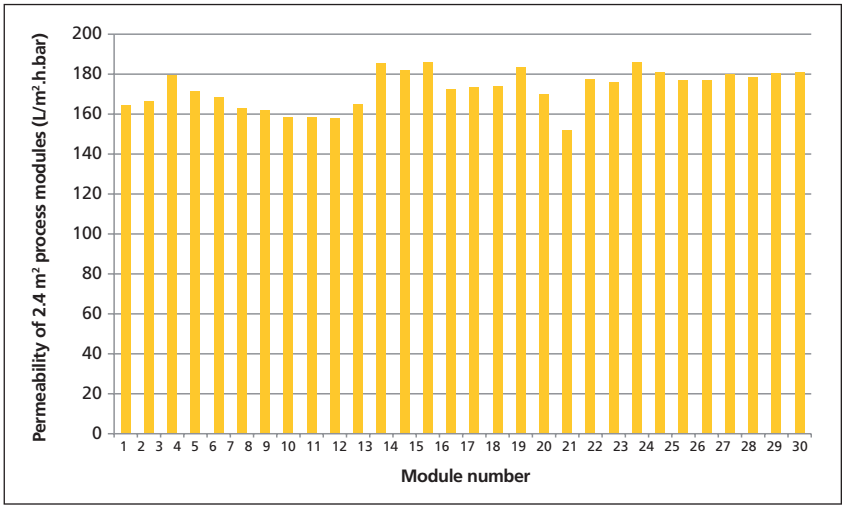



**Figure 2:** Water flow rate in Virosart HF capsules taken from three individual lots of the 2.4-m² module. Each capsule lot was built from a different membrane lot. The average permeability is 170 liter/m²h bar.




ALL FIGURES COURTESY OF THE AUTHORS

**Figure 3:** The performance of the Virosart HF module family was tested for scalability using the same batch of a buffered human IgG model protein stream (highly blocking) until 95% flux decay was achieved.

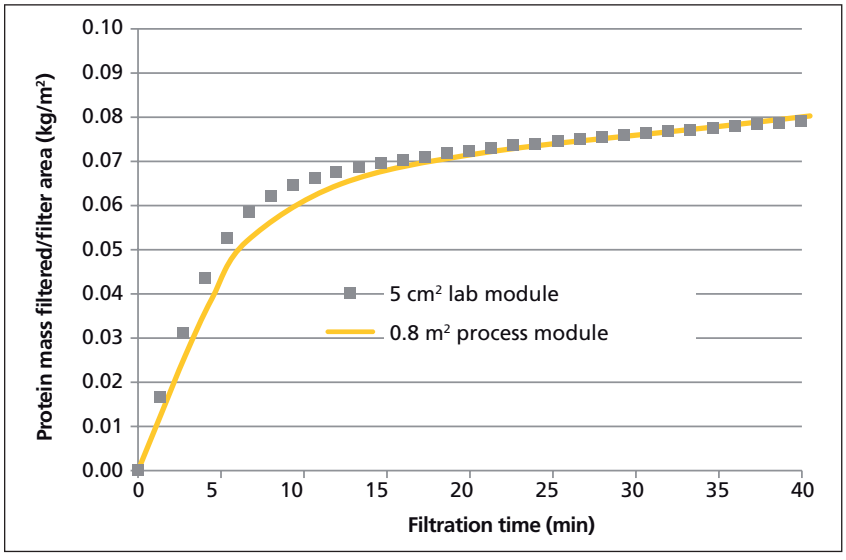
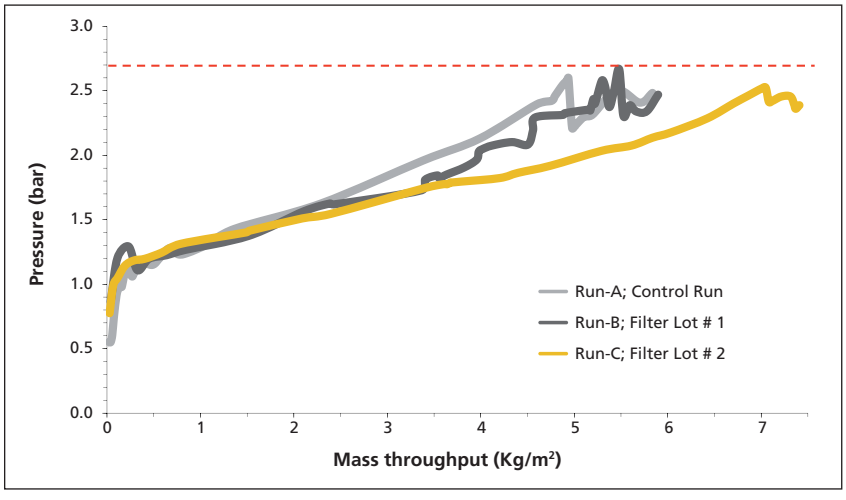



**Figure 4:** Virosart HF laboratory modules were challenged with a 10-20-g/L monoclonal antibody solution (pH 6-7, conductivity 4-8 mS/cm) spiked with 0.5% MMV. Experiments were carried out at constant flux at 120 L/m²h. The membrane was challenged with up to 7.4-kg antibody/m² resulting in a permeability decay of more than 70%. A log reduction value of greater than 5 was achieved in both spike trials (Run B and Run C).




on a case-by-case basis, that their virus clearance steps are acceptable and efficient.

## FILTER DESIGN AND VALIDATION

To overcome current limitations in virus filtration—and as a step toward the development of common standards—a high-performance, hollow-fiber parvovirus filter has been developed, which demonstrates robust retention at high transmembrane pressures. The unique structure of the membrane and its chemically modified surface address many of the limitations of current retentive filters.

The Virosart HF filter (Sartorius-Stedim) features a surface-modified asymmetric polyethersulfone (PES) hollow-fiber membrane optimized for the manufacture of monoclonal antibodies (see **Figure 1a**). The membrane is characterized by a funnel-like pore size gradient designed to achieve the robust retention of parvoviruses under challenging conditions (such as high blockage or pressure release) without impeding the efficient transfer of high-molecular-weight proteins such as monoclonal antibodies. The membrane is surface-modified with a hydrogel-forming, low-binding polymer, to reduce the adsorption of soluble proteins and protein aggregates. The pore size gradient and the hydrogel are unique aspects of the membrane that contribute to its high performance. The hollow fibers can be packed densely into modules ranging in capacity from 5 cm² to 2.4 m², the latter presented as a presterilized 10-in single-use device (see **Figure 1b**). The capacity of the filter can be extended by combining it with the Virosart MAX adsorptive pre-filter, featuring an optimized polyamide microfiltration flat-sheet membrane in a homogeneous triple-layer configuration, with a nominal pore size of 0.1 µm.

Validation of Virosart HF has proven the consistent performance of the product family. **Figure 2** shows selected validation data (permeability of 2.4-m² process modules) for illustration. However, measures have been taken to ensure future product quality from lot-to-lot. Validated in-process as well as release tests are performed during the manufacture and release of Virosart HF membranes and modules accord-

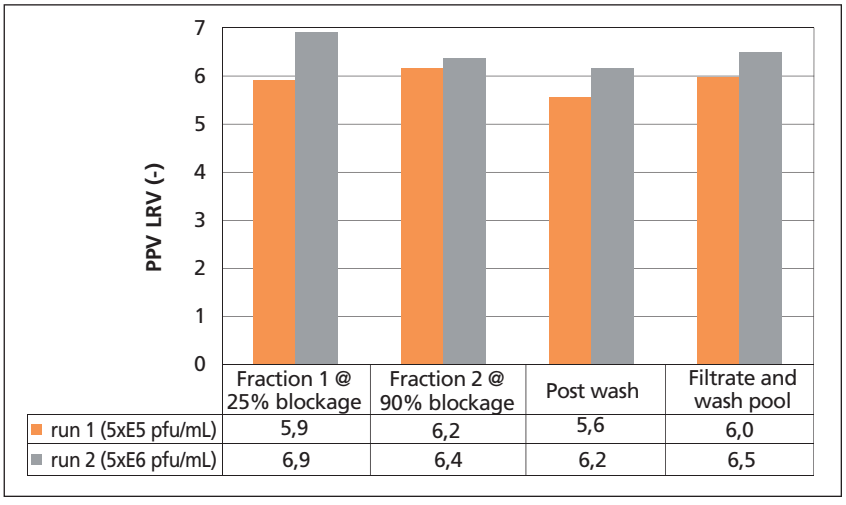
ing to pre-defined sampling plans to measure and monitor critical performance attributes of all product components.

Membrane testing includes in-process and lot release tests. Membrane performance release tests are executed on laboratory modules that have experienced the same manufacturing steps as laboratory or process modules that would be shipped to customers. Virosart HF modules are in-house integrity tested by air-diffusion as well as gamma irradiated. Three membrane release tests—bacteriophage PP7 retention in buffer, bacteriophage PP7 retention in human IgG (grab sample at 75% flux decay), and water permeability—are consequently performed on lab modules, which have also been flushed with water, dried, and then exposed to gamma irradiation. Protein filtration capacity is monitored while PP7 retention in buffered human IgG solution is determined. These release tests ensure that membrane performance items meet expected and validated levels.

In addition, Virosart HF modules are released based on a 100% inspection scheme. Water flow rate and integrity of each module is tested prior to shipment. Integrity testing is based on an air-diffusion test at 4.5 bar and modules subsequently released based on a correlation between diffusive flow rate and PP7 retention.

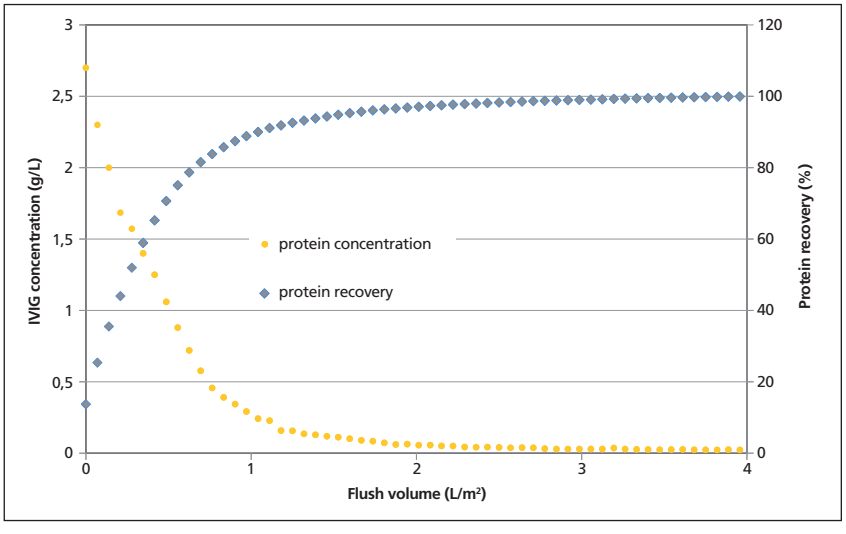
The performance of the Virosart HF module family was tested for scalability using the same batch of a buffered human IgG at 2 g/L. Three different 5-cm² laboratory modules and one 0.8-m² process module were challenged at 2 bar differential pressure until 95% flux decay was achieved (see **Figure 3**). The volume vs. time filtration data for the three laboratory modules was averaged and compared to the corresponding data for the process module according to the filtered protein mass per filtration area. **Figure 3** confirms that (based on the performance data gathered using 5-cm² devices) laboratory modules can be scaled-up to larger feed stream volumes and filter areas.

**Figure 5:** PPV retention in a human IgG model protein feed stream at different degrees of flux decay, during post wash and in the final filtrate and wash pool executed at two different spike levels.




| | Fraction 1 @ 25% blockage | Fraction 2 @ 90% blockage | Post wash | Filtrate and wash pool |
|---|---|---|---|---|
| run 1 (5xE5 pfu/mL) | 5,9 | 6,2 | 5,6 | 6,0 |
| run 2 (5xE6 pfu/mL) | 6,9 | 6,4 | 6,2 | 6,5 |

**Figure 6:** Analysis of the recovery flush of a 0.8-m² Virosart HF process module. The data shows that, already after 3 liter flushing per m² membrane area, 99% of protein is recovered.




The retentive capabilities of the Virosart HF filter were tested under worst-case conditions, by challenging with a 10-20-g/L monoclonal antibody solution (pH 6-7, conductivity 4-8 mS/cm) spiked with 0.5% MMV. Experiments were carried out at constant flux at 120 L/m²h. To implement worst-case load conditions, the membrane was challenged with more than 5.5-kg

**Table I:** Summary of the filtration and MMV retention data for the control trial (Run A) and the two spike trials (Run B and Run C).

| Run | Water permeability ($L/m^2h$ bar) | Final permeability ($L/m^2h$ bar) | Final permeability decay (%) | Mass throughput ($kg/m^2$) | MMV Log reduction value (-) |
|---|---|---|---|---|---|
| A | 190 | 42.1 | 77.5 | 5.8 | N/A |
| B | 184 | 39.2 | 78.4 | 5.9 | 5.19 |
| C | 162 | 43.5 | 72.7 | 7.4 | ⩾5.21 |

antibody/$m^2$ resulting in a permeability decay of more than 70%. Two spike trials and one control trial were conducted for comparison.

In all three trials, the transmembrane pressure increased over time but the pressure profiles varied slightly from run to run with the mass throughput ranging from 5.8 to 7.4 $kg/m^2$ (see **Figure 4**). The transmembrane pressure did not increase above 2.7 bar in any of the trials and thus remained below operating pressure. Breakthrough was observed in one of the spike trials but the other achieved complete retention. The log reduction values for the pooled permeate were 5.19 and 5.21, respectively (see **Table I**).

These data show that Virosart HF achieves robust log reduction values of greater than 5 even under challenging conditions, thus meeting the retentive requirements of a high-performance parvovirus filter with minimal lot-to-lot variability.

The Virosart HF was next tested with a feed stream comprising PPV spiked buffered human IgG solution at a IgG concentration of 0.1 g/L. Two spike trials were carried out with different amounts of virus, the first containing $5x10^5$ pfu/mL and the second $5x10^6$ pfu/mL. The log reduction values were determined using permeate grab fractions taken at 25% and 90% flux decay. The log reduction value was also determined in the post-wash fraction and the overall filtrate and wash pool. As shown in **Figure 5**, Virosart HF achieved robust log reduction values for human viruses regardless of the extent of blockage. The log reduction value for PPV was also high in the post-wash fraction, resulting in an overall pool log reduction value >6 in both tests.

Finally, the flush volume required to achieve the recovery of the target protein was determined by monitoring the permeate stream using an in-line UV-detector at 280 nm (see **Figure 6**). A 0.8-$m^2$ Virosart HF module was challenged with a 2-g/L buffered human IgG model solution until ~40% flow decay was observed, and then the module was flushed with buffer at 2 bar differential pressure. The protein concentration in the permeate stream was calculated from the UV measurement based on a previously determined calibration curve. Flushing the membrane with 3 $L/m^2$ achieved 99% protein recovery.

## CONCLUSION

The Virosart HF is a novel, high-performance parvovirus filter based on a surface-modified asymmetric PES hollow fiber membrane. Rigorous testing confirmed its consistently robust retentive properties and high filtration capacity under challenging conditions, including an inlet pressure of up to 5 bar, up to 90% blockage, loading of up to 7.4-kg antibody/$m^2$ and spiking with 0.5% PPV. The hollow fiber membrane can be packed densely into scalable modules with a small footprint ideal for single-use campaigns.

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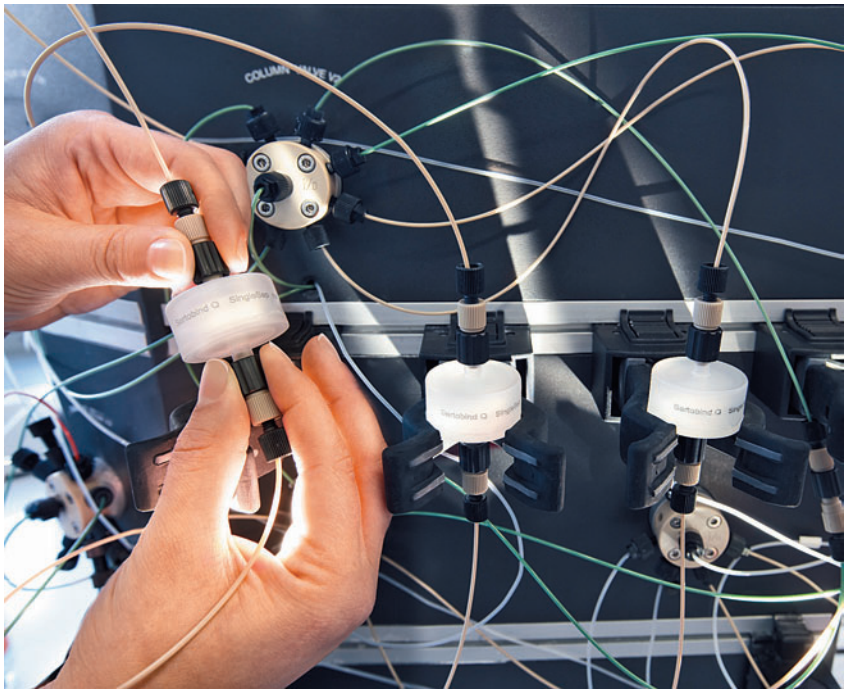

Photo Credit: Sartorius Stedim Biotech

# The Future of Downstream Processing

As constant scale up grows out of favor in the biopharmaceutical industry, new—and old—approaches are required. The author reviews the state of downstream processing and considers potential solutions, including the streamlining of full processes and borrowed technologies.

UWE GOTTSCHALK

The biopharmaceutical industry is becoming increasingly dependent on innovation and change to make progress in a commercial environment that simultaneously demands higher productivity, higher quality, and lower costs (1). Recombinant protein titers have improved from tens of milligrams to more than 10 grams per liter over the past 25 years, and at the same time, batch volumes have increased so that we face the prospect of batch yields exceeding 100 kg of protein in the next decade (2). Over the same period, regulatory demands have become more onerous (3) and the pressure to reduce costs has increased as more biopharmaceuticals come off patent and overseas manufacturers begin to take an interest in western markets (4). It is inevitable that biopharmaceuticals will at some point be regarded as commodities, and manufacturing on the ton scale will be necessary for certain products that are required in large, repetitive doses, such as topical antibody formulations.

Progress in the industry has been impressive, but most of the increases in productivity achieved in pre-

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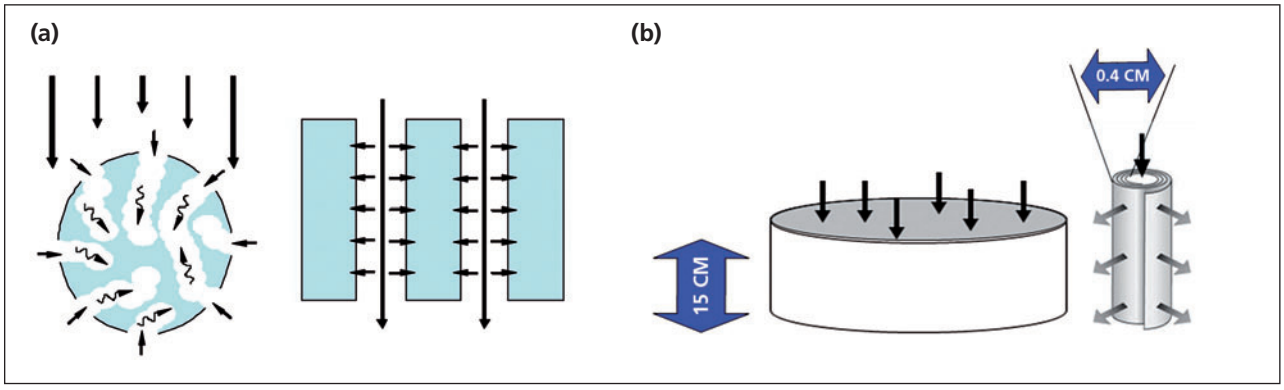
**Figure 1.** (a) Mechanistic comparison of solute transport in bead resins (left) and membrane adsorbers (right), where thicker arrows represent bulk convection, thinner arrows represent film diffusion and curved arrows represent pore diffusion. (b) Comparison of bed height in columns (left) and membrane adsorbers (right). Using membrane adsorbers is functionally equivalent to shortening columns to near-zero length, resulting in a similarly small pressure drop that allows extremely high flow rates, thereby reducing overall process times up to a 100-fold. In this example, both formats have a 1350 $cm^2$ frontal surface; the column has a bed height of 15 cm; and the membrane adsorber has a bed height of 0.4 cm. The height to frontal surface ratio is approximately 100 for the column and nearer to 3500 for the membrane device.




vious decades have resulted from improvements in the upstream production phase, with more efficient bioreactors and better media formulations sharing the limelight with cell lines that are intrinsically more productive because of the development of more effective screening technologies to identify the most productive clones (5). Downstream processing is now routinely found to be the bottleneck in biopharmaceutical manufacturing because its capacity has not kept pace with upstream production (1). This is largely due to the incremental nature of technological improvements in downstream processing, which do nothing to address the absence of economy of scale. Unlike upstream production, where a more productive cell line generates more of the product without increasing costs, the costs of upscaling downstream production are linear because a feed stream containing more of the product requires larger amounts of materials such as buffers and chromatography resins (i.e., higher titers), which translates linearly into higher manufacturing costs (6). The future success of downstream processing, therefore, depends on disruptive, game-changing innovations rather than incremental ones (1, 4). This need for innovation reflects the increased demand for biopharmaceutical products, the regulatory focus on quality in the manufacturing process, and the stratification of the market due to the advent of biosimilars or follow-on biologics (3).

## RUNNING TO STAND STILL

The first 15 years of biomanufacturing can be considered as a golden era, where manufacturers had the luxury of using inefficient processes because the product itself was far more important (3). Most biopharmaceuticals were required in small doses and demand was sufficiently low to allow plenty of slack in the system. It was also pointless investing in process efficiency when any tweaks and modifications would arouse the suspicious eye of regulators. It was better to let sleeping dogs lie and be satisfied with the status quo. In this environment, innovation was considered a burden rather than a bonus.

Inevitably, this relaxed attitude to process efficiency resulted in an immense amount of wastage because up to 50% of product batches failed to come up to specifications (3). To address this waste, FDA ordered that processes should be designed with quality attributes taken into account (7, 8). The process was no longer simply a means to an end to generate the product, but became part of the product. As the economic screws began to tighten and demand increased, so manufacturers turned to the age old strategy of scaling up their production to achieve cost savings, and this is where the industry began to flounder. Whereas upstream production can be scaled up almost indefinitely by increasing the productivity of cells growing in a bioreactor, downstream processing has limits imposed by physics and chemistry. Downstream processing is driven by the mass of product; therefore, increased productivity requires corresponding larger volumes of buffer, larger storage tanks

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and preparation areas, larger filters, and most importantly larger amounts of chromatography media. For the production of antibodies (i.e., where Protein A resin is typically used in the primary capture step), the costs of scaling up are in some cases greater than the extra revenue made possible by the increased upstream productivity. Manufacturers find themselves in the paradoxical situation that there is no longer an economy scale in manufacturing, but rather an economic depression reflecting the physical limits that constrain the size of the apparatus used in separations (e.g., chromatography columns and the associated piping, skids, and buffer reservoirs). So far the extra demand has been absorbed by contract manufacturers offering their spare capacity to fulfill quotas, but this is a short-term measure that cannot cope with the predicted increases in demand from hundreds of products currently in clinical development, all requiring at least pilot-scale manufacture according to GMPs (9).

How can this productivity dilemma be addressed? With constant scaling up no longer a viable approach, the industry must return to its roots and innovate to succeed. Manufacturers are currently considering three solutions, all inspired in some way by the more encouraging regulatory landscape that rewards rather than punishes innovation. These solutions are the streamlining of existing processes, the revisiting of simple technology solutions currently employed in the bulk chemical industry, and the use of innovative technologies from the bleeding edge of biopharmaceutical research. These latter technologies have the potential to introduce game-changing processing options into an industry still mired in technologies that were state-of-the-art 20 years ago. On a cautionary note, however, technologies from the bleeding edge can fail, and the rash adoption of new and untested technology platforms can punish the eager company seeking innovative solutions. This is the new dilemma in downstream processing.

## STREAMLINING AND REDESIGNING AN EXISTING MANUFACTURING PROCESSES

Many processes for biopharmaceutical manufacturing were designed at a time where process efficiency was considered unimportant (3). More recently, manufacturers have sought to increase the efficiency of each unit operation, but they are only now starting to consider redesigning the entire process train to see if cost savings can be made through streamlining the process as a whole. The trend towards process streamlining owes a lot to FDA's quality-by-design (QbD) principles, which themselves derive from the design-of-experiments (DOE) concept. QbD considers experimental design as a landscape with peaks of efficiency and troughs of inefficiency. Similarly, the design space of a manufacturing process is littered with efficiency peaks and troughs, but there is not always a simple path leading upwards to the most efficient process. Therefore, process design incorporating efficiency and quality from first principles involves going back to the drawing board and evaluating the critical attributes that contribute to an efficient process.

Most companies are now applying these principles and actively streamlining their processing strategies wherever possible. Antibodies take center stage because they represent more than half of all biopharmaceutical products in development and their common properties mean that it is possible for companies to share process efficiency data that are applicable across platforms (10, 11). It is for this reason that antibody manufacturing has benefitted from the development of so-called generic platform processes, which are broadly similar for all antibodies but can be tweaked to match the specific properties of individual products (12).

Antibody manufacturing provides an excellent example of the application of process redesign and streamlining principles to increase productivity, cut costs, and maintain product quality. Most manufacturers use three chromatography steps for antibody purification, starting with a very expensive Protein A capture step that is placed immediately after clarification, followed by anion exchange (AEX) chromatography in flow-through mode to extract negatively-charged contaminants such as host cell protein (HCP), endotoxins, host DNA, and leached Protein A, and then either cation exchange (CEX) chromatography or hydrophobic interaction chromatography (HIC) in retention mode to remove positively-charged residual contaminants and also product related impurities such as aggregates and degradation products (13). Modern platform processes also serve as orthogonal strategies for virus removal.

Realizing that no further cost savings could be gained by scaling up the aforementioned process, Pfizer explored the design space around the standard process and found that certain modifications could reduce costs considerably without impacting on the quality of the antibody (14). They introduced two types

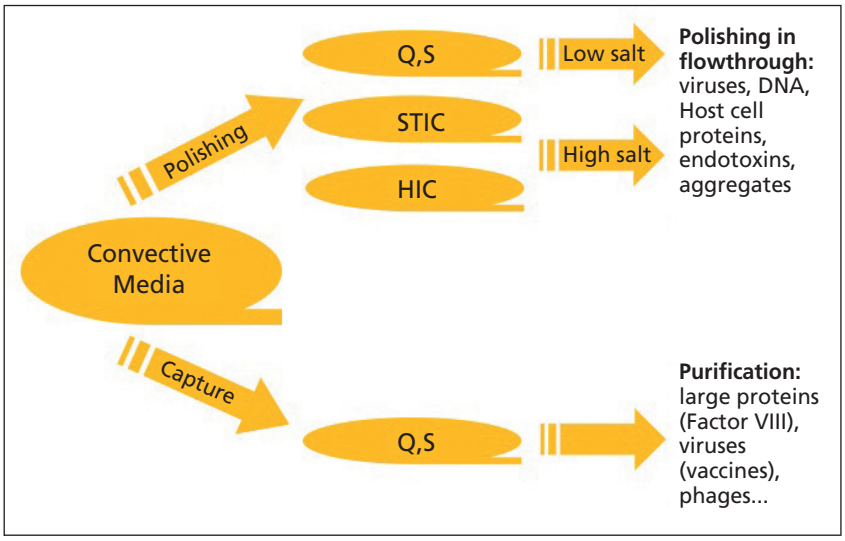
of process modifications, one in which the order of the polishing steps was reversed and another in which different separation technologies were used to increase process capacity (i.e., using membrane absorbers for the flowthrough chromatography step and replacing the depth filtration step with continuous centrifugation) (15). These changes increased the efficiency of purification to such an extent that, for some antibody products, the cation exchange step became unnecessary, reducing the process from three columns to two columns or even a single column. Not only did this save the direct costs of column resin and buffers, but also reduced the process time by >45%, which doubled the productivity in terms of batch processing (14).

## LOOKING WITH A FRESH EYE AT OLDER TECHNOLOGIES

The capacity crunch in downstream processing has been avoided or overcome in other industries by adopting simple and inexpensive technologies (16). In the bulk chemical industry, the conventional pharmaceutical industry, and the food and detergent industries, expensive processing solutions such as chromatography would never be considered because the costs of implementation would not be sustainable in these high-volume, low-margin processes. Is it possible for this simple approach to be applied also in biopharmaceutical manufacturing?

Several recent developments suggest that simpler technologies could indeed find a niche in biopharmaceutical manufacturing, particularly in the early processing steps where the complex mixture of particulates and solutes have the most potential to foul expensive membranes and resins (16, 17). Tangential flow microfiltration, depth filtration, and (continuous) centrifugation are the current methods of choice for the clarification of the feed stream, and one or more of these processes may be employed in series to remove larger particulates until finally a polishing depth filter or dead-end filter can be used to remove fines and thus reduce feed stream turbidity (18). Efficient and inexpensive clarification becomes more challenging with higher-titer cell culture processes because these are characterized by a greater cell density and often a longer process time, resulting in a higher solids content, more particle diversity (i.e., size and physical properties), and most challenging of all, a greater proportion of fine particles that escape coarse filtration. A technology that is widely employed in the beverage industry and also in wastewater processing is the use flocculants to link small particles together and create easier-to-remove aggregates. Flocculation is achieved using polymers that bind simultaneously to the surfaces of several particles through electrostatic interactions, creating larger particles that may sink under gravity or may be removed more easily by centrifugation or filtration. In the bioprocessing industry, flocculation has been used to help remove whole cells from fermentation broth, but more recently it has also been used to remove fine cell debris and proteins. A simple and inexpensive strategy recently applied in antibody manufacturing is the creation of a calcium phosphate precipitate by adding calcium chloride to a final concentration of 30 mM and then potassium phosphate to a final concentration of 20 mM. Precipitation traps cell debris in larger particles, allowing removal by centrifugation for 10 min at 340 x g and yields a clear supernatant with the recovery of ~95% of the antibody (19). Interestingly, this strategy also removes some soluble host cell proteins and nucleic

**Figure 2.** Selection guide for convective media, such as membrane adsorbers. HIC is hydrophobic interaction chromatography. STIC is salt tolerant interaction chromatography.



acids. The beauty of flocculation is that it does not introduce any additional impurities to the feed stream, because the flocculant is removed along with the aggregated particles.

Precipitation is widely used as a purification approach in the bulk-chemical industry, and given that precipitation can be induced by simple changes in the environment (e.g., varying the temperature or pH, increasing the salt concentration [salting out], or adding organic solvents), it should be easy to apply the same principles in bioprocessing (20). Precipitation has, therefore, been used to remove soluble impurities from the feed stream during antibody manufacturing, and these solids can then be trapped by filtration or pelleted by centrifugation leaving a clear feed stream relatively enriched for the target protein (20). In an innovative adaptation of this approach, the antibody itself can be precipitated under mild conditions and recovered from a collected pellet thus removing many contaminants in a single step (21). This is possible because the mild precipitation conditions allow the protein to be redissolved without loss of activity. Several groups have developed methods to precipitate antibodies in large-scale processes, and this could replace Protein A chromatography in the long term (22, 23). Precipitation methods using n-octanoic acid are used for the removal of contaminants in at least two industrial antibody-manufacturing processes (24, 25).

In the final purification steps, another traditional technology being considered for use in biopharmaceutical manufacturing is crystallization. This technology involves the separation of a solute from a supersaturated solution by encouraging the growth of crystals. The crystallization process involves the formation of a regularly-structured solid phase, which impedes the incorporation of contaminants or solvent molecules, and therefore, yields products of exceptional purity suitable for the preparation of pharmaceutical proteins, coupled with the realization that protein crystals enhance protein stability and provide a useful vehicle for drug delivery (26). Protein crystallization has been developed into a commercial technology for drug stabilization and delivery and several current manufacturing processes involve crystallization including the production of recombinant insulin, aprotinin and Apo2L (27).

## GAME-CHANGING INNOVATIONS

Although process redesigns and traditional technologies can contribute to the development of downstream processes, they provide only incremental improvements that marginally increase process efficiency. Incremental or evolutionary technologies have been the mainstay of the bioprocessing industry for the past 20 years, and column chromatography provides one of the best examples of this phenomenon in action (28). These slow marginal gains, however, are already beginning to decline and [the industry is] reaching the stage where it is becoming difficult to envisage how sustainable processing can continue without a major injection of downstream processing capacity. One way this can be addressed is to embrace genuinely novel technological approaches that change the rules of the game. Companies that survive on innovation populate the fringes of the biopharmaceutical industry, and some of these innovations are disruptive in the sense that their influence on the industry is unpredictable and could contribute to a radical change in bioprocessing.

Most technological innovations in bioprocessing have been incremental, but there are several recent examples of disruptive innovations that have challenged the established business model and caused real grassroots change in the industry. Again, many of these changes have affected upstream productivity first (e.g., disposable bioreactors and buffer/media storage bags), but there are examples in downstream processing (e.g., the introduction of simulated moving bed chromatography, expanded bed chromatography, monoliths, and membrane adsorbers) (1, 29). These innovations have taken hold in niche markets but are now beginning to adopt mainstream positions. Disposable modules for downstream processing occupy a more mature status in the development cycle (30). The use of disposable filter modules is now an industry standard, but these are being complemented in more and more processes by disposable membrane adsorbers and innovative combinations that exploit both adsorption and size exclusion as orthogonal separative principles (31, 32).

Disposable anion-exchange membrane adsorbers are replacing traditional flow-through chromatography steps for polishing, particularly the removal of host-cell proteins, nucleic acids, and viruses, because of their high flow rates compared to packed resins and the absence of cleaning and validation requirements (32-34). The performance advantage of membranes over resins reflects the transport of solutes

to their binding sites mainly by convection, while pore diffusion is minimal (see **Figure 1a**). These hydrodynamic benefits increase the flow rates and reduce buffer consumption compared to columns, thus shortening the overall process time by up to 100-fold. Polishing with an anion exchange membrane can be conducted with a bed height of 4 mm at flow rates of more than 600 cm/h, providing a high frontal surface area to bed height ratio (see **Figure 1b**). However, a more diverse range of surface chemistries is now available (see **Figure 2**). Membrane adsorbers, therefore, are also challenging the hegemony of column chromatography in other biomanufacturing steps, such as bind-and-elute capture steps (35), hydrophobic interaction chromatography (36), and even salt-tolerant chromatography in high-conductivity buffers (37), which broadens the polishing window as shown in **Table I**. Membrane absorbers have been substituted for both flow through and bind-and-elute polishing steps during the manufacture of various commercial products. These devices are also increasingly viewed as ideal for virus clearance because they interact with both large and small, and both enveloped and non-enveloped viruses, and can easily be combined with other concepts such as irradiation with ultraviolet light (UVc) and dead-end filtration (38, 39).

**Table I:** Broader polishing operation window with salt-tolerant membrane chromatography.

| | Sartobind Q | Sartobind STIC |
|---|---|---|
| Protein Binding [g/L]<br>BSA in 200 mM NaCl (20 mS/cm) | 3.6 | 36 |
| DNA Binding [g/l]<br>DNA in 50 mM NaCl (7 mS/cm) | 7.3 | 22 |
| LRV with Mouse Minute Virus (MMV)<br>Fraction 1, 150 mM NaCl<br>Fraction 2, 150 mM NaCl | <br>2.10<br>1.81 | <br>3.82<br>> 4.96 |

The flexibility of disposable modules and their capacity to integrate into any stage of the production process is arguably their most important benefit. This reflects the broad industry perspective that manufacturing flexibility is now perhaps at least as important as capacity considering the large numbers of products in clinical development (1,4). Process development can be streamlined and expedited because different modules can be tested in various combinations to arrive quickly at the best overall set of process options, and the absence of cleaning and validation requirements can shorten the time required to develop a finalized process by months or years. The ability to replace each module completely also makes it easier to assemble process trains for new products in existing premises without cross-contamination and to achieve the ideal concept of continuous integrated bioprocessing (40). Continuous integrated bioprocessing has been implemented in upstream production using profusion cultures (41–43) and, more recently, in a series of linked downstream operations (44–46). Only in the past two years, however, have serious efforts been developed to link upstream and downstream components into a single unified continuous process (40, 47).

## WHAT DOES THE FUTURE HOLD?

Innovations that take into account not only the current state of the industry but also future challenges and demands are likely to be the most successful in the long term, but bleeding-edge technologies always come with risks that must be evaluated by manufacturers looking at major investments into capacity. The perceived bottleneck in downstream processing can be addressed with lower-risk approaches such as streamlining current production processes, with moderate-risk approaches such as introducing technologies that have already proven suitable in other industry settings, or with higher-risk approaches involving the incorporation of novel technologies. In several cases, these novel technologies have already proven their credentials in several processes. Companies following the paths set by the first adopters, the trailblazers of the industry, can be assured that the technologies involved now have established their credibility.

The future of biomanufacturing is likely to rely more on innovation and flexibility than on traditional strengths such as large facilities and the financial muscle to invest in them. Disposable manufacturing is likely to play an increasingly important role as companies maneuver in a crowded market to protect their R&D investments while more and more generics become available. The ability to scale up or down quickly, to switch to new campaigns rapidly, and to produce multiple products in the same facility will be a key metric of success. The future of bioprocessing will require the industry

players to embrace the need to change. In the words of US Congressman Bruce Fairchild Barton, "When you are through changing, you are through."

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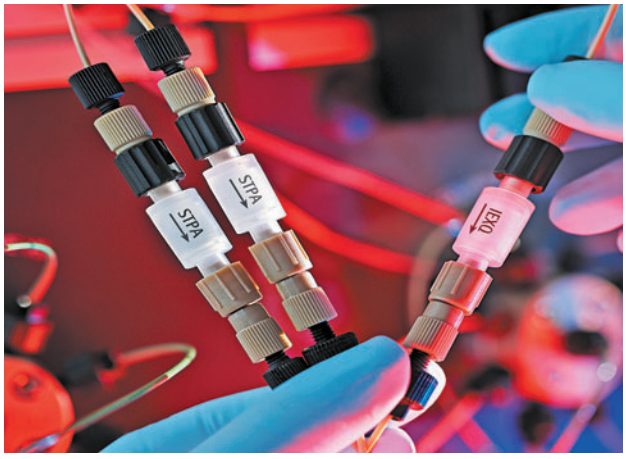


Photo Courtesy of Sartorius Stedim Biotech

# Protein Purification Using Single-Use Technology

## Uwe Gottschalk, vice-president of purification technologies, Sartorius Stedim Biotech, discusses specific challenges in protein purification.

**BioPharm:** What are the challenges to developing a reliable, robust means of performing protein purification in a single-use unit? What properties would a single-use product for protein purification need to be economically viable?

**Gottschalk:** Industry is changing to a 'market-pull' scenario, mainly due to regulatory pressure to proactively provide best practice. Single-use manufacturing adds value in certain downstream unit operations. While such practice has never been questioned for steps such as virus or sterile filtration, we are in the middle of that shift in chromatography and X-Flow filtration. Although it can be demonstrated that single-use strategies provide better process economies, their main advantages stem from factors such as accelerated development timelines and risk mitigation.

**BioPharm:** What recent developments in membrane adsorbers could lead to single-use technology for protein purification? Could membrane adsorbers replace packed-bed column chromatography?

**Gottschalk:** Membrane adsorbers offer two main advantages compared to packed-bed chromatography: the fluid dynamics of a convective media that can process large feed-stream volumes with extremely high flow rates, and large pore sizes that provide accessability and thus high dynamic binding capacities for large molecules such as DNA and viruses.

As a result, single-use membrane chromatography devices are typically much smaller in size and require only about 5% of the original buffer volume. The sweet spot for membrane chromatography is related to these two stand-alone features and it shines in areas like contaminant removal (polishing in flow-through mode) and purification of viral vaccines. In these applications they start dominating the industry's development platforms and will take over from resins completely. Recent developments include salt-tolerant chemistries on membranes that bind viruses under physiological conditions (no in-process dilution requirements).

**BioPharm:** What technologies in development could make protein chromatography a continuous process?

**Gottschalk:** In general, continuous processing offers the advantage of higher productivities, from a smaller footprint to an advantageous process economy and chromatography. Technologies such as simulated moving-bed chromatography have the potential to decrease column sizes because they use the total binding capacity as well as the overall lifetime of the chromatography medium. In this setup, a single-use design is possible if, for example, the same sample of medium is recycled within the purification of just one batch of product. Although this scenario would cut costs during clinical manufacturing, it is probably less beneficial in routine manufacturing and questions of scale up remain. **BP**

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