The term **preparative chromatography** means different things to different people. In QA/QC laboratories, it means purifying samples for further analysis. In a clinical setting, it means purifying them for diagnostic purposes. On manufacturing floors, it means separation and purification of a product from the mixture in which it is produced. The common element is purification. Whereas resolution, detection, and analysis are most important to analytical chromatography (see Chapter Two), here the key is recovery. As the “Size Matters” box shows, HPLC is used everywhere in biotechnology. Many of the same issues important to analytical LC come into play for preparative and process chromatography. But in purification processes, contamination is more than an annoyance that can distort detection results; getting rid of it is the whole point.

**CONSIDER THE SOURCE**
Different products present different issues when it comes to purification, and in many cases chromatography is the only solution for removal of problematic impurities.

**Gene therapies** (oligonucleotides) are either synthesized chemically (like most small peptide drugs) or produced by microbial cell culture as discrete plasmids. Coming from enzymatic and solid-phase extraction processes, the products of chemical synthesis are easier to purify than biological products. Impurities are solvents, unreacted intermediates, catalysts, degradants, and leachables, all different enough from the product — whether peptide or nucleic acid — that separating it from them is relatively straightforward. HPLC, supercritical fluid, and membrane chromatography are common choices, and here is where simulated moving bed (SMB) chromatography shines.

Chiral separation of enantiomers is an application of SMB that has proven very useful for semicontinuous oligonucleotide purification (1). Because SMB works only on simple binary mixtures, it is very good as a final purification step to separate “right-handed” and “left-handed” mirror-image versions of the molecule. With stereoisomers such as these, only one version (usually) is biologically active. A common example is thalidomide, which might never have caused the birth-defects it’s infamous for had chiral separations been around back in the 1950s and 1960s. It turns out that one enantiomer of the drug was the adverse-effect culprit, and the other was the therapeutic. Unfortunately the molecules are otherwise identical, so separations methods of the time could not distinguish between them.

**Vaccines** come in a variety of forms: whole organisms (weakened or killed), acellular or subunit vaccines, toxoid molecules and conjugates, DNA-based vaccines,
and therapeutic vaccines (2). The type of vaccine determines what sorts of separations can be performed, of course, but the source is what determines which impurities must be removed. DNA-based vaccines are treated as gene therapies, above. Therapeutic vaccines are usually proteins but may be gene therapies as well. Subunit and toxoid vaccines are usually proteins (see below), and whole organisms are grown in cell culture, so they’re subject to many of the same issues. Some whole viruses can be separated from cell culture supernatant by chromatographic means, but intact bacteria are too big for that. Conjugate vaccines may combine more than one of those types, and the combination process can introduce separations challenges of its own.

**Proteins** vary in size and complexity, but they’re all much bigger and more complex than chemically synthesized “classical” pharmaceuticals. As the sole expression of genomic information, often further modified by other cellular mechanisms, proteins are the basis of all the variation we see in living things. A trip to the coast here in Oregon will give you a great perspective on just how much molecular variation we’re talking about: The real differences between the individual creatures in a tidepool, the seabirds flying overhead, the trees and ferns in the forest nearby, and the human observer enjoying it all are the proteins at work in and among the cells in their bodies. Enzymes, antibodies, hemoglobin, insulin — all are proteins, and it is their interaction with each other and with smaller organic molecules that keeps a living thing alive. Such variation makes protein chemistry an exciting scientific endeavor, and it makes purification of therapeutic proteins particularly challenging to those involved in drug development. Not only must the particularities of a given molecule be taken into account at every stage, but the nature of its source will make a great difference in the kinds of impurities that must be dealt with along the way.

**From Microbial Sources:** Products that come from microbial fermentation are the simplest biologicals to process (3). Bacteria produce toxins and pyrogens (which can cause adverse reactions if patients come into contact with them), and they sometimes play host to bacteriophage viruses (which must be removed even though they aren’t known to affect humans or animals). Many yeasts and other microbes will secrete the recombinant products they make to their circulating medium, greatly simplifying its purification. Separating the product from the cells, in that case, is a simple filtration or centrifugation step. If, however, the product is not secreted, the cells will need to be disrupted (usually by grinding because microorganisms have tough cell walls), and then all their contents will have to be separated from the resulting feedstream. That includes DNA, RNA, and cellular debris.

**From Cell Culture:** Animal cells — whether mammalian or insect cells — require specialized media in which to grow (4), and the source of ingredients used can be of great concern in downstream processing. Whereas microbes grow on simple mixtures of sugar, water, and a few specialized nutrients, animal cells need complex mixtures that mimic their natural environment (in the tissues and/or circulation of living animals) as closely as possible. Trends in cell culture media are moving away from real animal sources for those ingredients because the associated troubles they can bring (prions, in particular) are too difficult and/or expensive to process away.

Cell cultures, themselves, can and do become infected with bacteria or viruses. Unlike bacteriophages, many of these pathogens are known to infect human beings. So they are of vital concern in downstream processing. Luckily, they’re also much larger than the protein products, so size-exclusion chromatography can often help remove them.

**From Transgenic Animals:** Proteins of therapeutic value can be produced in the milk of transgenic goats or other mammals, as well as in the eggs of transgenic hens (5). Each source presents its own particular purification concerns. Milk is full of native proteins, fats, and sugars — as well as microorganisms. And egg whites may be simpler mixtures (roughly 10% proteins and 90% water), but the impurities are all proteins themselves and thus can present obvious challenges in processes meant to separate them from the therapeutic molecule. Eggs, too, may be infected by pathogenic microbes. Viral clearance, in particular, is a major concern with the products of animal sources, whether transgenic whole organisms or cultured cells.

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**SIZE MATTERS**

**Capillary HPLC (nanoscale):** miniaturization of analysis using very small volumes of solvent at 100–1000 nL/minute flowrates, used for rare/costly materials and high-throughput analysis of many different samples

**Analytical HPLC (microscale):** column IDs range 1–4 mm, with flowrates up to 50 mL/min; ubiquitous in biotech laboratories

**Semipreparative HPLC (small-scale):** purifies and isolates milligram to gram quantities for analysis and development scale-up/scale-down studies

**Process HPLC (large-scale):** column IDs range up to a meter or more, and 100 L/minute flowrates for separation and purification of products from large-volume feedstreams in manufacturing operations

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SUPPLEMENT

From Transgenic Plants:
Therapeutic proteins may be expressed in the leaves, seeds, roots/tubers, or fruits of transgenic plants (6). Like transgenic animal work, this technology is still new enough that it isn’t clear which system will win out in the end. But purification processes are being developed for both. The downstream processing concerns differ here depending on the specific plant species and means of expression. Questions regarding the vagaries of soil composition come up unless the plants are grown hydroponically. And it is not yet known which plant pathogens will present the most trouble. Sure, we eat fruits and vegetables all the time. But we’re not injecting their extracts directly into our veins — which is the most common delivery method for protein therapeutics. Part of our digestive system’s purpose is to protect the body from microbes and viruses on the food we eat, so whatever we administer through bypassing that safety mechanism had better be very very pure.

And achieving “very very pure” results is much more possible when chromatographic steps are involved in a downstream manufacturing process than when they are not. In fact, without them it’s just about impossible.

Removal of What?
As shown above, different sources impart different types of contaminants to a product feedstream. And different LC unit operations are used to remove those various impurities and adventitious agents. Here are some examples of great concerns in downstream processing.

Endotoxin Removal: Exotoxins (toxic molecules secreted by bacteria) were identified in the 1800s. Later, Gram-negative bacteria were shown to be toxic in themselves, and the cause was termed endotoxin: a lipopolysaccharide (LPS) complex found in the outer membranes of bacterial cell walls. LPS binds to immune system cells (macrophages and dendritic cells) and triggers inflammation. Endotoxins are a problem with any bacterial-sourced product, but especially when the product is not secreted but must be harvested from homogenized or ground bacterial cells. Cation-exchange chromatography is often the answer (7). However, endotoxins bind very strongly to those anion exchangers, which complicates their removal from DNA preparations (gene therapies) and highly acidic proteins. For DNA purification, reversed-phase chromatography (often in the form of HPLC) is often the best choice. Anion-exchange chromatography sees some use for this purpose too (8).

Nucleic Acids As Contaminants:
More often than not, however, DNA is considered a contaminant rather than a product, especially in

Figure 1: Just as in astronomy it is impossible to show relative sizes (of planets and stars) and distances (in astronomical units and lightyears) to scale on the printed page, so too is it difficult to represent the differences in things on the very small scale of molecules and cells. In (A) below, molecular sizes are compared using Dalton (Da), kiloDalton (kDa), and nanometer measurements. One Dalton is equal to the molecular weight of a hydrogen atom.

In (B) below, microorganisms and cells are compared using measurements on a larger scale, from nanometers to micrometers.
products of animal cell culture. Animal cells are fragile and seldom secrete recombinant proteins into their surrounding medium (4), so all their contents become an issue in purification. DNA is of particular concern because it can be expressed by cell machinery (in patients, for instance) into proteins (even foreign ones). Also of concern are messenger and transfer RNA, which are its intermediates for translation and expression. Ion-exchange membrane chromatography is showing promise in the removal of nucleic acids (which have a high negative charge), as well as endotoxin and viruses.

Viral Clearance: As illustrated in Figure 1, virus particles represent about the size limit (so far) for chromatographic separations. Filtration is the most common method of dealing with them. But expanded-bed chromatography has made their removal by LC from process feedstreams easier, and membrane chromatography is beginning to be applied as well. Protein A affinity chromatography is the common choice. Viral clearance is vitally important with all products made by animal cell culture or transgenic animals. In fact, BioProcess International is planning a special supplement issue devoted to the topic this fall.

Process Intermediates: Certain chromatographic processes can introduce their own contamination, particularly those used for removing difficult substances. What they get rid of, themselves, makes the extra step that must follow worth the trouble. For example, bound metals can alter the surface electrical charge, solubility, and hydrophobicity of proteins, even making them clump up (aggregate) in solution. Proteins are highly reactive with certain metals (9), which is the basis of immobilized-metal affinity chromatography (IMAC). “If you are using IMAC or if your product exhibits elevated tendencies toward metal complexation, you will need to have an affirmative strategy for metal ion elimination. Otherwise metal contamination will be an uncontrolled variable in your process, and a proximal cause for potentially crippling process variation” (9). One method for removing metal ions involves very precise proteolysis (10). Another is addition of ethylenediaminetetraacetic acid (EDTA) with imidazole, histidine, or histamine to process buffers as metal chelators in a displacement elution step.

Special Hardware Issues

Columns are, of course, central to LC systems. Whether glass, acrylic, or stainless steel, they must not “leach” contaminants or react with the media, buffers, and solutions inside them. Chemical resistance is a necessity, especially in HPLC operations. Glass and acrylic columns allow visual examination of their contents, which can help process engineers troubleshoot during process development. At full process scales, glass becomes impractical because of its weight and relatively low strength. Strong and lightweight, shatter-resistant acrylic becomes necessary for column IDs larger than about 40 cm. However, it is less chemically resistant (particularly with organic solvents and certain cleaning agents) and has historically presented leachables trouble. Polymer manufacturers, however, have responded to those complaints by developing better acrylics for use in chromatography column construction.

Stainless steel columns, however, are most often used at large scales (IDs 0.5–1.5 m or even larger). Because of its strength and chemical resistance, stainless steel is traditionally the most common material found in biotech manufacturing (11). It is vulnerable only to sodium chloride (cooks are familiar with the “pitting” effect of salt on their stainless steel pots) and certain acids in long-term exposure. Even glass and acrylic columns use stainless steel fittings and structural supports. A woven form is the most common frit material, supporting the column packing but allowing solvents and buffers to flow through. Process temperature control is achieved with chillers or heat exchangers of stainless steel construction. For GMP processes, all materials must be fully traceable to their source; vendors of LC equipment and supplies are obliged to provide accurate documentation of their own manufacturing processes as well. The technical documentation they provide will be used in qualification, process validation, maintenance, and regulatory submissions. HPLC columns are pressure vessels, so they must meet certain design standards (of the American Society of Mechanical Engineers and the European Pressure Equipment Directive, for example) for operator safety. The European Union requires pressure relief valves as part of an LC system.

Cleaning: Sanitary conditions are critical to all pharmaceutical manufacturing processes. For efficient clean-in-place procedures, LC columns need good flow paths without dead spaces. The best designs improve column efficiency, as well, by preventing peak tailing and band broadening (as discussed in Chapter Two) that can occur with backwash and other flow problems.

Packing and Flow: Analytical columns often come prepackaged with specialized media, but process-scale LC is more economical when media are bought in bulk and packed into columns on-site. The methods used depend on the media. Operator training and experience has always made a big difference in manual packing methods, but here is another area where automation is changing things. For this discussion, I turn to my “Chromatography Advisor,” Pall Corporation’s Ian Sellick (see box, next two pages).

Capacity and Overload: The more efficient a column separation, the lower its maximum sample loading capacity (13). Columns can be lengthened to increase their loading capacity, but then process designers increase either their flow rate (which reduces efficiency) or media particle size (again reducing efficiency).
Higher flow-rates increase sample throughput, and larger particles work better at lower pressures than do smaller particles. It is a delicate balancing act, so the maximum sample volume must be determined. At what volume will loss of resolution exceed an acceptable level? Economics enters the picture here, where in strictly analytical chromatography it would not be much of an issue.

So in preparative LC, column capacity is approached differently (14). Volume overload is defined here as “loading that no longer permits the isolation of product at the desired purity or recovery levels.” This takes into consideration all molecules in the sample and stationary phase because they all compete for active binding sites. When process chromatographers discuss capacity, they do not mean the capacity factor (see Chapter Two), which measures retention.

“The goal of a preparative purification is the maximum production of purified product per injection” (14). Over time, impurities from previous samples build up in reused columns, even those washed with solvent in the regeneration step. If the cost of solvent used to regenerate a column is higher than for new packing or column replacement, then it will make sense to replace the matrix or column instead. “Often, the first few centimeters of a column suffer the most contamination, and removing this material and replacing it with fresh packing can be performed relatively easily” (14). One special technique for ensuring purity is heartcutting, which means collecting only the middle portion of a peak of interest and discarding potentially overlapping portions — not an economical choice, so it’s used almost exclusively in small-scale semipreparative chromatography with less-costly samples. Another technique, recycling, increases the effective length of a column by reinjecting the eluent back into the top of it, sometimes repeatedly. Or two preparative columns can be connected by multiport valves and alternated in an automated procedure (14).

**Fraction Collectors** take the place in importance of analytical detectors used in preparative chromatography (Figure 2). Detectors are present, but they cannot be too sensitive because their purpose is to identify when to start the fraction collection (13). They do need to tolerate high flow rates and must not impede the flow of eluent. In bioprocessing, UV–Vis detection (set to 280 nm wavelength, as explained in Chapter Two) is commonly used.

In fraction collection, a multiport valve is connected to a series of collection vessels. The detector can

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**THE ADVANCING SCIENCE OF COLUMN PACKING**

Excerpted from Ref. 12

Several years ago, packing chromatography columns was largely a manual process that required extensive skills and training akin to a practiced art. “Dry” or “tap” packing was the norm: Resins were supplied dry, columns agitated to help particles settle, and the media then were hydrated directly in the column. When resins began to be supplied prehydrated, “flow packing” became the new and improved procedure. Operators would pour media as a diluted slurry directly into columns from a container or conditioning tank. The suspended media were then stirred to ensure as much homogeneity as possible. A column’s top end cell was inserted and sealed, then liquid flow pressure was applied to settle the resin particles. The end cell would be manipulated to remove any air trapped above the column bed, while the bed surface had to remain undisturbed. Given the manual manipulation required, reproducing such flow-packing procedures in a cGMP environment was typically beyond the skills of even moderately experienced operators. Since the mid-1990s, an automated method called “pack-in-place” has changed things, transforming production-scale column packing from a special craft into a hard science by largely eliminating the human factor. Today, about 70% of all new process chromatography operations use some type of pack-in-place automation. New innovations make monitoring and control more accurate and reliable. All this has improved packing reproducibility and process control to ensure more consistent packed bed performance overall.

Pack-in-place procedures are selectively calibrated for different chromatographic media and conditions. The goal is optimal media compression within a homogenous bed to maximize column performance. By eliminating operator handling, automation removes the human variables of manual packing methods, vastly reducing the chances of column failure. It also improves manufacturing productivity because column packing and unpacking both take place with the columns fully assembled.

**Eliminating Contamination:** Fully contained systems may be the greatest benefit of pack-in-place. Adjustable or fixed-end cells are in place at ultimate bed heights, and operators form beds by pumping in slurry and simultaneously exhausting excess liquid. All procedures, including clean-in-place (CIP) operations, occur without removal of the top column assembly. That minimizes the risks of operator contact with potentially hazardous materials (in vaccine production, for example) as well as exposure of drug products to external contamination. So a pack-in-place procedure is more hygienic than traditional manual packing methods.

A key feature of automated packing is the separate slurry packing system/station (SPS), which delivers media slurries to columns during packing and unpacking (Photos 1...
be programmed to signal a selector valve, which chooses between them. The more complex the feedstream, the more ports and vessels are needed. Volatile solvents used in RPC can be evaporated from the collected fractions, or buffer exchange/removal procedures (sometimes additional LC steps in themselves) are used after or between unit operations.

Validation and Qualification:
Regular instrument calibration and qualification are essential to GMP operations. Design, installation, and operational qualification tests are generally performed by LC system vendors and the people involved with setting up a new laboratory or manufacturing suite (15). Performance qualification is (usually) a yearly test of pumps, autosamplers, detectors, and other hardware to ensure that an instrument continues to operate as specified. Depending on the size of a biotechnology company, performance qualification is typically the responsibility of its QA/QC or regulatory compliance group. Most important, “the confirmation of proper system performance is incomplete until it has been fully documented” (15). Data presented in tabular format identifies the equipment tested and tests run with dates and signatures of the people responsible. As with laboratory instrumentation, out-of-specification results trigger corrective and preventive actions (CAPAs): Appropriate repairs and other corrections are made, then tests are rerun until their results are acceptable. To prevent unnecessary CAPAs, standard operating procedures (SOPs) should be explicit and unambiguous.

When it comes to qualification of LC columns themselves, industry experts recommend carefully choosing appropriate metrics, focusing particularly on column packing methods, and analysis of testing procedures to reduce errors and unnecessary CAPAs (16).

### The Advancing Science of Column Packing

Continued

and 2). An SPS system first mixes and then pumps media into columns at a controlled rate and pressure. Adjustable end cells can be set to the desired packed bed height. Media is pumped into the column to form a bed while excess buffer is exhausted.

To Each Its Own: The three general categories of chromatography media used for processing biotherapeutics are carbohydrate-based matrices (such as agarose, dextran, or cellulose), polymeric media (usually methacrylic or styrenic matrices), and rigid packings (such as silica or controlled pore glass). Packing procedures generally fall into one of four categories to accommodate them:

- Variable and pressure packing is used for moderately compressible media. The column pressure and bed height are allowed to increase slowly, and packing is complete when hydraulic pressure within the bed drops noticeably.
  - Constant-pressure packing is used with more rigid media, requiring that packing pressure be kept at a pressure exceeding process levels throughout a packing process until the bed meets its support.
  - Flow-compression packing involves setting the piston at a higher bed height as slurry is pumped in. The slurry is introduced and then the bed formed by flow-packing at pressures exceeding process levels, then the piston is moved onto the top of the bed.
  - Fixed media-volume packing is useful for costly media such as Protein A. The required media volume is premeasured in the slurry tank. Exiting mobile phase can be recycled to wash the slurry tank back into the column.

**Flow Requirements:** A goal for any chromatographic operation is to ensure optimal flow distribution within the column. Quality controllers want proof that a process is reproducible. Well-packed column beds show homogenous density throughout their surface area, with a controlled interstitial volume (spaces between the particles). As a liquid mobile phase enters a column, the flow profile should ideally form a ‘plug-flow’ pattern (the advancing face of liquid traveling through the column remains perfectly flat and horizontal where flow is vertical) with minimal band broadening. Broad bands during separation reflect poor resolution and performance, adversely affecting the purity of the target products being eluted. All parts of the liquid should travel at the same speed to prevent tail formation. Chromatographic media used vary, and some can be quite costly. Possible failures include density inconsistencies or local voids in the resin bed, which can lead to flow aberrations and irregularities and even to bed instability. In worst-case scenarios, beds can crack or form a moving void as feed is processed, ruining batches applied to the column.

**Figure 2:** Preparative chromatography set-up (dashed lines indicate flow of data, solid lines indicate flow of solution)

**ADAPTED FROM REF. 13**
Leachables must be identified and tracked, identity and porosity tests must be developed for chromatography media, and function testing should show why a given matrix was chosen. Protocols are written for hardware, packing, operations, and personnel.

Reproducible column packing (as discussed in the “Chromatography Advisor” box) helps to predict repeated success in purifying a molecule of interest. Before automated packing procedures, detailed protocols were the only way to ensure reproducibility from one column to the next — and from one operator to the next. The better (more robust) a packing method, the more consistent and reproducible were the associated qualification test results. Because packing is not yet automated everywhere, it is still a critical issue in the validation of most downstream processes. Procedures are optimized for each type of packing used, and test methods are chosen for accuracy, reliability, and flexibility. Function tests may be used as well, but testing is expensive and need not be excessive.

Qualification testing is for equipment and hardware. Validation testing performs the same function with methods, whether for purification or analysis (17), as Chapter Two discusses in more detail.

**Scale-Up and Process Economics**

Decisions regarding bioprocess technology must include cost as a consideration. To most business people that is obvious, but it is not always obvious to the people working on a manufacturing floor — or in an analytical laboratory. Even seemingly small costs can add up: labor, cleaning time, consumables, and so on. But they can make the difference between a choice that doesn’t make economic sense and one that does. Economics are more of an issue at larger scales, of course, whether that means higher volumes (as in commercial production) or more samples (e.g., discovery efforts). Scaling up small volumes from a few samples to many is pretty straightforward: You need automated, high-throughput methods. Scaling up from the process development laboratory to the manufacturing floor, however, can present real challenges.

When it comes to bioprocess development, time-to-market looms large in importance. The old saying that “time is money” very much applies in the biotechnology industry. Biotech drugs and diagnostics often address previously undetectable or untreatable conditions (or those with few other options available), so the market for a given indication can be fiercely competitive, with more than one drug in development. The first one to clear all the scientific, regulatory, and economic hurdles between drug candidate and marketed therapeutic will gain a competitive advantage over any others that follow. It has been said that each day lost can cost a company millions in potential revenue — and many thousands in operating costs.

**Scale-Up/Scale-Down:** That commercial advantage can be lost if scale-up complications create more hurdles to slow time-to-market. So for downstream process development, unit operations must maintain all their operational parameters throughout the various sizes from laboratory to pilot to production scale. Linearity is thus vital to scaling up chromatographic unit operations.

As shown in the “Size Matters” box, chromatography columns regularly used in biotechnology range in size from a few centimeters to two meters in diameter. The larger the column ID, the more chance that problems will arise: mainly pressure limitations, packing defects, and flow irregularities. Product purity, recoveries, and batch-to-batch consistency can be detrimentally affected. Some specific problems that may be encountered include differing impurity profiles of the larger scale production process, accumulation of impurities in reused columns, and stresses placed on the drug substance during solvent evaporation steps following fraction collection.

This takes us back to the mathematics of chromatographic theory described in Chapter Two. Linear column scale-up means that feedstream pressure and velocity stay constant throughout, thus allowing consistent quality of separations for all volumes. And the math has to work out for each step upward in scale. As mentioned, an increase in sample volume will lead to decreasing resolution. “Few things produce a worse sinking feeling than watching a chromatogram develop during scale-up and realizing that it doesn’t parallel the standard you have to match” (18). Sometimes instrumentation may be to blame; other times, the types of aberrations mentioned above are the culprits. Some optimization and scale-up issues associated with the various types of chromatography used in biotechnology — size exclusion, ion exchange, hydrophobic interaction, and affinity — are discussed in their respective sections of Chapter One.

For pharmaceutical research (product development), typically only a few milligrams to grams of the target molecule are needed in pure form (19). Gravity alone controls the flow rates at small analytical and semipreparative scales. A certain amount of automation becomes necessary at larger scales: pumps, valves, and computer
controls (as described above). At the pilot scale, certain variables are set: sample loading methods, separation times, solvents used, washing and regeneration steps, for example. “The main goal of method development is a simple, well-automated, and robust separation process able to run 24 hours per day. A separation process that is too sophisticated might turn our sleeping time into night shifts” (19).

Table 1 lists the basic unit operations involved in scaled-up purification of three well-known biopharmaceuticals (20). They are discussed in much more detail by the source.

Solvents present a particular concern during scale-up. Whereas the solid phase is usually regenerated and reused, “often the major operating cost in preparative LC is the solvent rather than the packing material” (19). Organic solvents are more expensive than water, of course, but some of them are cheaper than others. Acetonitrile is the most costly, methanol the least. Solvent may be recovered during evaporation and reused, but only in pure rather than mixed form.

Scale-down studies are performed to test unit operations already in place — e.g., to prove their viral clearance ability (18). They are just what they sound like: rather than scaling up by moving to larger and larger columns, a unit operation is scaled down — that is, performed at a pilot or analytical scale to provide test data without requiring large, expensive sample volumes. Such scaled-down versions must be validated to represent the full-scale process step.

Making Process Changes: Once validated, a chromatographic method cannot be changed — even if “better” materials and/or instruments show up on the market (21). Right? Not exactly. If the cost of revalidating a new method will be offset by long-term savings, then the change may be made. If the necessary materials are no longer available, then the method must be changed no matter what. And if the original method is actually found to be flawed — not likely if it’s been validated — then it will certainly need to be revisited. However, if the overall process within which this particular unit operation fits is producing material that has already been approved for market by one or more regulatory agencies, then making process changes is a much more complicated issue.

It can be done — sometimes. In the United States, postmarket changes fall into three categories: class one changes (likely to have a major impact on the safety, potency, or efficacy of a product), class two changes (expected to have an intermediate impact), and class three changes (little or no affect on the final product) (22). Class one changes incur a great deal of trouble and expense because they may be implemented only with regulatory approval of supporting data submitted by a company’s regulatory affairs department. Class two changes can be made 30 days after such data are submitted unless otherwise instructed by the FDA. And class three changes require no immediate reporting. Companies must be careful in defining a proposed change. Anything that will require new clinical studies is class one, as are all changes to product specifications. Replacing equipment with similar (but not identical) equipment counts as class two. So do minor increases in production scale. Changes made to improve GMP compliance (in response to new Points-to-Consider documents, for example, or new pharmacopeial standards) fall under class three.

**FUTURE TRENDS**

Membrane chromatography may be the most exciting development to come along for process chromatography in many years. Vendors such as Pall Corporation (www.pall.com) and Sartorius AG (www.sartorius.com) are combining membrane filtration with ion-exchange chromatography to address issues of speed and process economics. The surface of microporous membranes are treated to the same chemistries as found on the surface of chromatography resins, and the membrane acts as one of those theoretical plates we discussed in Chapter Two. Stack them and you can scale up without the diffusional flow complications of deeper column beds.

In column chromatography, the surface area for binding is mostly contained within the pore structure of the resin and thus accessible only through diffusional

| Table 1: Step-by-step purification at the manufacturing scale (ADAPTED FROM REF. 20) |
|---------------------------------|---------------------------------|---------------------------------|
| **Somatotropin**               | **Alpha-Interferon**            | **Tissue Plasminogen Activator** |
| Fermentation                   | Fermentation                    | Cell culture (serum-free conditioned media) |
| Centrifugation                 | Centrifugation                  | Zinc chelate chromatography     |
| High-pressure homogenization   | Homogenization                  | Concavavalin A affinity chromatography |
| Pellet wash                    | Extraction                      | Dialysis                        |
| Solubilization                 | Dilution                        | Lyophilization (and interim storage) |
| Renaturation                   | Microfiltration                 | Dissolution                     |
| Microfiltration                | Concentration                   | Centrifugation                  |
| Concentration and diafiltration| Filtration                      | Size-exclusion chromatography   |
| Anion-exchange chromatography  | Immunoaffinity chromatography   | Lyophilization (and interim storage) |
| Hydrophobic-interaction |
| chromatography                 | Concentration and diafiltration | Dissolution                     |
| Concentration and diafiltration| Cation-exchange chromatography  |                                              |
| Microfiltration                | Concentration                   |                                              |
|                               | Size-exclusion chromatography   |                                              |
Methacrylate monoliths were first described in 1990, and the first CIM products became available in 1998. So a wealth of experience has accumulated in applying this technology to biomolecular discovery and production and in quality assurance/validation activities. The medium is a short, continuous, and homogenous methacrylate or styrene-divinylbenzene rigid polymeric block cross-linked with uniform, three-dimensional, interconnected channels with 62% porosity. Macrochannels are about 1500 nm in diameter, and mesochannel diameters are >100 nm. The larger channels contribute to a column’s low back pressure, even when operating at elevated flow rates; the smaller ones provide a large surface area for high dynamic binding capacity. CIM monolithic columns are resistant to chemical and thermal stress. Each monolith can withstand temperatures of 4 °C (39 °F) to >120 °C (248 °F), the working pH range is 1–14, to >120 °C (248 °F), the temperature of 4 °C (39 °F) the monolith can withstand thermal stress. Each resistant to chemical and CIM monolithic columns are dynamic binding capacity. The supports have an extremely high capacity for very large molecules, ensuring that they can easily permeate the matrix. These features decrease the purification time by an order of magnitude, which in turn reduces purification costs. Therefore CIM supports combine the separation power, capacity, and sample distribution of conventional porous particle chromatographic columns with the convective mass transport of membrane technology.

CIM monolithic chromatographic supports can be prepared for analytical and QA use (disks, minidisks, 96-well plates, and syringes) and for production (tubes) (Table 1). The analytical disks can be used for proteomics, screening, and as precolumns for MS–MS. The tubes are used for preclinical, pilot, and full cGMP production. The process of scaling up from screening to small-scale purification, pilot-scale purification, and then production is facilitated by the identical performance and purification profiles of all short monoliths (no matter what size or shape). Once method optimization is completed, scale-up from the laboratory to full-scale production takes only a few days, potentially resulting in significant time and cost savings.

Both small and large columns are easy to operate and adjustable to meet the needs of biochromatographers. It takes seconds to set up or change the configuration of a column, and the columns have a fast equilibration and regeneration. There is no packing of particles, as is necessary with conventional columns, and no need to worry about air bubbles because they are not trapped in the monolithic structure but are washed out with the mobile phase. The separation quality does not change with the speed of the separation. The support has no diffusion limitations because the flow-through channels make all pores accessible to the biomolecules. They operate at high flow rates in all chromatographic modes on analytical and preparative scales. They are compatible with any peristaltic pump or conventional LC/HPLC or FIA system.

The working lifetime of a CIM support varies depending on the sample purity. One thousand injections of a standard protein mixture were performed with no loss of resolution or dynamic binding capacity, and the support was regenerated after every 200 injections. CIM supports come in a complete range of chemistries, including ion-exchange (anion, cation), reverse-phase, hydrophobic-interaction, affinity/immunoaffinity, and epoxy. A CIM CDI (1,1-carbonyldiimidazole modification) and a CIM IMAC (immobilized metal affinity column) support will soon be available. The ability to bind a wide range of enzymes, immunoglobulins, and peptides makes them ideally suited to serve as bioreactors, as affinity columns, and for on-line and off-line in-process control. The existing chemistries have applications for virus purification, virus concentration, and vaccine production; pDNA production, monoclonal antibody isolation; biomolecule discovery; purification of basic and acidic proteins; and oligonucleotide and peptide separations and bioconversions.

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forces. Large molecules and viruses cannot diffuse into the pores, so they are limited to binding on the outer available surface area of the resin. With no diffusional flow limitations, membranes offer a 10- to 100-fold higher capacity per unit volume than resins for DNA, viruses, plasmids, and other substances. And membranes are linearly scaleable, which is a critical consideration for manufacturers moving their products from laboratory-through pilot- and on to production-scale processing.

Membrane chromatography comes in the form of disposable capsules or cartridges. Small laboratory-scale devices are used for proof-of-concept studies and for protocol optimization. They can be added to existing process cycles, usually as a polishing step. Disposable capsules eliminate the need for cleaning and associated validation, which speeds processing and reduces labor and buffer costs. (23)

Another important development in chromatography is the use of ultrasound technology to aid in column packing. “Ultrasound detectors are sensitive to bed compression, mobile-phase composition, and the presence of soluble components in the chromatography bed. They detect real-time bed formation data that can be recorded and verified before a column is used. When combined with dynamic feedback control from a packing station, ultrasound helps ensure consistent and correct packing before a column is used” (23). Although it is a technology still in development, it could improve packing procedures by providing real data where before there was only guesswork.

As described in the “Monoliths” box, monolithic columns are a more established technology. Their high permeabilities allow them to operate at high flow rates while still exhibiting good efficiencies and sample capacities (24).

If the past is any indication, the future of chromatography will be in greater automation, improved resins, and ever more sensitive detection. For process chromatography, the successful vendors will be those who continue to listen to their customers and respond to their needs for ease of scale-up and ever more economical processes.

REFERENCES