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New concepts in the chromatography of peptides and proteins

Douglas D Frey[1] and Xuezhen Kang[2]

Although chromatography using a variety of novel bed configurations (e.g. fluidized beds, expanded beds, simulated moving beds, annular rotating beds, etc.) has been of recent interest, the majority of practical applications of analytical and preparative chromatography employ a stationary adsorbent bed into which a feed slug is charged periodically, similar to the technique first described by Mikhail Tswett over 100 years ago. However, new concepts in both the practice and theory of fixed-bed chromatography are continuing to expand the available range of applications for separating peptides and proteins.

Addresses
[1] Department of Chemical and Biochemical Engineering, 1000 Hilltop Circle, University of Maryland Baltimore County, Baltimore, Maryland 21250, USA
[2] Genzyme Corporation, One Mountain Road, Framingham, Massachusetts 01701, USA

Corresponding author: Frey, Douglas D (dfrey1@umbc.edu)

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Introduction

Chromatography employing a stationary adsorbent bed is one of the most widely used methods for separating biomolecules because of its predictability, simplicity of operation, and ability to achieve high resolution. Chromatography is also unique among bioseparation methods in its scalability, with applications ranging from lab-on-a-chip devices where the column volume is as small as 100 nL to the process scale where column volumes larger than 100 L are commonly employed. This article reviews several concepts in the field of peptide and protein chromatography that have become important recently, and that pertain to chromatography performed on a variety of scales.

Monoliths

Although packed beds containing discrete particles (i.e. particulate beds) have historically been the column configuration most widely used for chromatography, recent advances in polymer science have made continuous beds of porous polymer, or 'monoliths', practical alternatives for many applications.

Synthesis and structure

Silica monoliths are produced by polymerizing a monomer, such as tetramethoxysilane, in the presence of a porogen, such as poly(ethylene glycol), so that a phase separation occurs during polymerization to produce a network of macropores. This is followed by a solvent exchange and leaching process, which produces a network of smaller mesopores inside the silica skeleton. Columns produced in this way have been recently introduced commercially by Merck KGaA (Darmstadt, Germany), under the trade name Chromolith, and by Phenomenex (Torrance, CA). These columns contain 2 μm macropores, which constitute the 'through-pores' in which fluid convection takes place, and 13 nm mesopores. They also incorporate either a C8- or C18-bonded surface phase and, because of the relatively small size of the mesopores, they are primarily useful for the separation of molecules no larger than moderately sized peptides [1–3].

Monoliths formed from organic polymers have also recently been commercialized, two examples are the Convection Interaction Media (CIM) introduced by BIA Separations (Ljubljana, Slovenia) and the UNO monoliths introduced by Bio-Rad Laboratories (Hercules, CA). CIM monoliths consist of a co-polymer of glycidyl methacrylate and ethylene dimethacrylate and are composed of polymer particles with a diameter of 0.6 μm, which are agglomerated together to yield a structure with 1.5 μm macropores. These monoliths are available in sizes ranging up to 800 mL and with a variety of surface chemistries, and have been used for the chromatography of molecules as large as genomic DNA consisting of 2300 base pairs (molecular weight 1.5×10^7 Da) [4,5,6•]. UNO monoliths consist of a co-polymer of acrylic acid and *N,N′*-methylenebisacrylamide. They have 1.5 μm macropores and are also available with a variety of surface chemistries. Additional types of organic polymer monolithic columns are available from Isco (Lincoln, NE) and Dionex/LC Packings (Sunnyvalle, CA; Amsterdam, The Netherlands). Note that the various monoliths described here can be considered to be variations of 'perfusion' chromatography column packings where convection in large intraparticle pores inside a particulate bed augments intraparticle diffusion, except that in this case a single perfusive particle occupies the entire column.

Theory

A dimensionless performance parameter that is especially useful for monoliths, as it does not involve a particle

diameter, is the 'separation impedance', defined by $E = \Delta P\ t_0/(N^2\ \mu)$, where ΔP is the applied pressure, t_0 is the elution time for an unadsorbed eluite, N is the number of theoretical plates (i.e. the retention time squared divided by the variance of the eluite band), and μ is the fluid viscosity. Accordingly, a lower value of E corresponds to better performance in terms of the resolution that can be achieved for a given pressure drop and separation time. By incorporating Darcy's law (i.e. $Q/A = K_D\Delta P/(L\mu)$, where Q is the volumetric flow rate, A is the column cross-sectional area, L is the column length, and K_D is the Darcy's law permeability) and the plate height (i.e. $H = L/N$), and as the total porosity of the column including both the macropores and mesopores is given by $\varepsilon_T = LA/(Q\ t_0)$, it follows that $E = \varepsilon_T\ H^2/K_D$. Although in past work the separation impedance has mainly been applied to the case of small molecules and analytical-scale columns, in this review this parameter will also be applied to large molecules such as proteins and to columns intended for preparative-scale use.

An equivalent hydraulic particle diameter for a monolith, d_h, can be defined as the particle diameter that yields the measured permeability when using the Blake-Kozeny equation with $\varepsilon_e = 0.4$, which is a typical interparticle porosity for a packed particle bed. It follows from this definition that $d_h = 29\ K_D^{1/2}$, so that the separation impedance can also be written $E = 840\ \varepsilon_T\ (H/d_h)^2$. Furthermore, $\varepsilon_T \approx 0.75$ for a large variety of monolithic and particulate columns, in which case it can be concluded that the square of the reduced plate height, $(H/d_h)^2$, is proportional to E. In general, H varies with the flow rate and binding affinity, so that to a first approximation the minimum value obtainable in practice, H_{min}, can be used to compare different columns. As the preceding relations also apply to a particulate column if d_h is replaced by the average particle diameter in the bed, d_p, it follows that the separation impedances of monoliths and particulate columns can be evaluated on a relative basis simply by comparing H_{min}/d_h for a monolith to typical values of H_{min}/d_p for a packed bed. For a microparticulate packing where $d_p < 10$ μm, and when the eluite is either weakly retained or entirely unretained, H_{min}/d_p generally lies in the ranges of 2 to 4, 2 to 5, and 5 to 10 for small molecules, peptides and proteins, respectively [7]. These ranges therefore provide a basis to which H_{min}/d_h for a monolith can be compared.

Performance comparisons

The performance of Chromolith columns and related silica monoliths have been studied by several workers [8–11,12•,13•,14•]. These investigations indicate that H_{min} for eluites small enough to freely access the monolith mesopores is approximately 8 μm and the permeability is 7.2×10^{-14} m^2, which corresponds to $d_h = 8$ μm. Therefore, $H_{min}/d_h = 1$ and E_{min} is one-quarter of the value observed in a well-packed particulate column for these types of eluites. However, in some cases plate heights were also found to increase substantially at higher flow rates, for larger eluites, and under conditions of high retention, so some performance limitations may exist.

Jungbauer and co-workers [15,16] used linear gradient elution chromatography and frontal chromatography under mass-overloaded conditions to determine that the plate heights for retained proteins in a CIM monolith range from 15 to 30 μm as the flow rate is varied. Plate heights for unretained proteins in an UNO monolith have been found to be in the higher range of 100 to 200 μm [17]. As the permeabilities of UNO and CIM monoliths are reported to be 3.9×10^{-14} m^2 and 1.1×10^{-14} m^2, respectively [17–19], it follows that $d_h = 6$ μm and $H_{min}/d_h = 17$, and $d_h = 3.2$ μm and $H_{min}/d_h = 5$, respectively, for these two columns. This indicates that H_{min}/d_h, and therefore also E_{min}, for a protein in an UNO column are somewhat greater than the typical values for a particulate column, whereas for a CIM column these parameters are smaller than those observed for the best-performing particulate columns. In addition, as H/d_h was observed in the above studies to increase relatively slowly with the dimensionless reduced velocity (i.e. with $v_f d_h/D$, where v_f is the interstitial fluid velocity and D is the eluite diffusion coefficient), it may be more advantageous to employ a monolith than indicated simply by its relative E_{min} value, especially for very large and therefore very slowly diffusing eluites. Finally, because of the way they are manufactured, it is easier to produce a monolith than a particulate bed with a bed width greatly exceeding its length for applications where this geometry is beneficial.

Although the results described above indicate that monolithic chromatography columns are highly promising, more work is needed to fully evaluate their potential. For example, additional comparisons are needed of particulate columns and monoliths that are intended for preparative chromatography, such as CIM columns, when both types of columns are used with mass overloading, as the greater equilibrium adsorption capacity of a particulate column may partially compensate for its other comparative deficiencies under these conditions. Finally, alternatives to monoliths that retain some of their useful features, such as rolled fabrics or membrane stacks, should be investigated as substitutes for certain applications [20,21].

New applications of theory

Chromatography has historically attracted the attention of many notable theoreticians, and it continues to provide a rich source of theoretical problems — the solutions of which often yield new insights into the method.

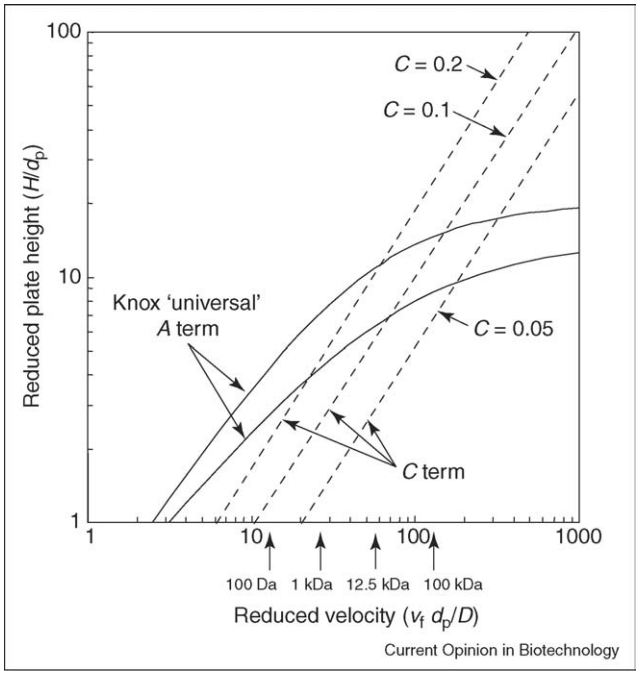
Interparticle fluid-phase dispersion

One theoretical problem that has persisted over many years concerns the proper form of the 'A' term in the van

Deemter equation, which is the equation describing the several contributions to the plate height. This equation is generally written in dimensionless form as $H/d_p = A + B/v + C^{\bullet}v$, where v is the dimensionless reduced velocity described previously and the A term, which is a function of v, accounts for interparticle (or interskeleton) fluid-phase dispersion. Recently, Knox has re-examined a large body of data and proposed a new 'universal' A term that applies to both particulate and monolithic columns [22,23••]. In these same studies Knox concluded that, in contrast to widely held opinions, the band broadening of small molecules is nearly always determined by the A term, as opposed to the 'C' term (which accounts for diffusion in the stagnant fluid that fills the particle pores) or the 'B' term, which is virtually always negligible in practice.

The observations of Knox are pertinent to this review for two reasons. First, the A term proposed by Knox on the basis of experimental data for packed columns and monoliths yields much larger plate heights, even at moderate flow rates, than the theoretical values corresponding to perfectly arrayed spheres or to a uniform monolithic structure. This implies that a significant reduction in plate height can be achieved by developing improved column packing methods that yield more efficient geometrical particle arrangements or by improved polymer synthesis methods that yield a more efficient arrangement of the macropores in a monolith. Second, although Knox considered mainly small molecules in his conclusions, if the A term that he proposes for microparticulate silica is presumed to be valid for larger molecules as well, then for conditions typical of high-resolution, gradient-elution high-performance liquid chromatography (HPLC) (i.e. $d_p \leq 4$ μm) this term might also be significant, and perhaps even dominant, for molecules up to ~50 kDa in size (as illustrated in Figure 1). A similar conclusion for the high-performance size-exclusion chromatography of synthetic polymers was also reached on a qualitative basis by Knox [22] and, more recently, by Popovici and Schoenmakers [24]. However, to reduce the magnitude of the A term in practice requires a better understanding of the dispersion processes in the fluid phase. Fortunately, new theoretical methods are under development that have the potential to provide this needed understanding, as described below.

Figure 1



Comparison of the reduced plate height contributions (H/d_p) from interparticle fluid-phase dispersion, as described by the Knox 'universal' A term, and intraparticle diffusion as described by the C term, as a function of the dimensionless reduced velocity ($v_f d_p/D$) for high-resolution HPLC. The two A term curves are the upper and lower limits of data fitted by Knox for various microparticulate silica packings. The C values used are typical for weakly retained small proteins so that the comparison shown is representative of protein gradient elution chromatography. Calculations also correspond to $d_p = 4$ μm, $\Delta P = 350$ bar, $L = 10$ cm, $\varepsilon_e = 0.4$ and $\mu = 0.01$ poise. The interstitial fluid velocity was calculated to be 0.14 cm/s using the Blake-Kozeny equation. Arrows denote the reduced velocities that apply to 100 Da, 1 kDa, 12.5 kDa and 100 kDa eluites with assumed diffusivities of 5×10^{-6}, 2.5×10^{-6}, 1×10^{-6} and 5×10^{-7} cm^2/s, respectively.

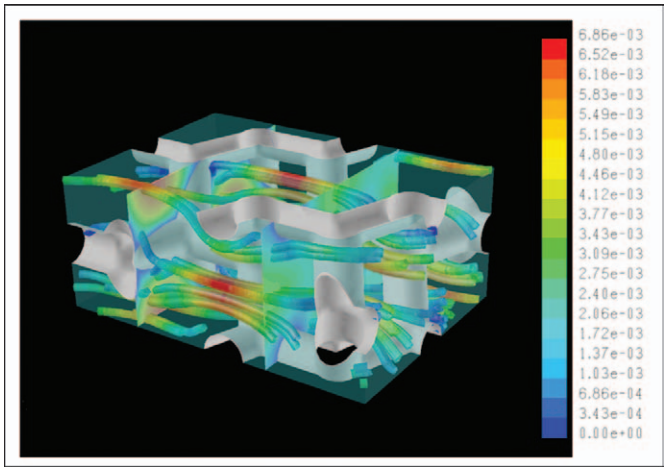
Computational fluid dynamics

Recently, computational fluid dynamics (CFD) has provided a more fundamental view of band broadening in both particulate and monolithic beds that could ultimately lead to the manufacture of more efficient columns by reducing fluid-phase dispersion. The CFD approach involves first solving the full Navier-Stokes equation for the flow field in a column, and then determining the behavior of a molecule undergoing convection and diffusion in this flow field. In one approach by Desmet and coworkers [25,26•,27••,28••], commercial CFD software was used to investigate two-dimensional arrays of cylinders as well as a three-dimensional model structure of a silica monolith (see Figure 2). Among other results, these workers have used CFD to better quantify the effects of nonuniform particle arrangements in particulate beds. Their results also suggest that the plate heights currently observed for silica monoliths can be reduced by a factor of nearly three simply by making these monoliths more uniform in structure, even if the size of the macropores is fixed.

Control theory

To facilitate the development of new pharmaceutical products, regulatory authorities have recently encouraged the use of innovative manufacturing approaches in the pharmaceutical and biotechnology industries. As summarized in several recent reports [29–31], it has been concluded that quality cannot be tested into products, but

Figure 2



Streamlines in a silica monolith model structure determined by computational fluid dynamics. The different colors denote the fluid velocities as indicated. (Figure provided courtesy of Vervoort and co-workers, Vrije University, Belgium.)

instead it must be engineered into the manufacturing process. Consequently, in recognition of their traditional hesitancy to embrace new technologies because of regulatory uncertainty, pharmaceutical and biotechnology companies are now being encouraged to use modern manufacturing techniques, such as process analytical technology (PAT) and multivariate statistical process control, even in the early stages of process development.

Consistent with this trend toward more innovative manufacturing technologies, Nagrath *et al.* [32•] have developed a novel run-to-run, statistical control strategy for nonlinear preparative chromatography where process outputs (e.g. the production rate) are controlled in the presence of both sporadic disturbances (e.g. variations in feed composition) and autocorrelated disturbances (e.g. variations in column efficiency). The method makes use of a detailed 'physical' chromatography model to initialize a simpler 'empirical' model. Parameters for the empirical model are then updated periodically using process data obtained from each chromatographic run, and the updated model is used in a model predictive control algorithm to accomplish the control tasks. The method was shown to account for the disturbances most likely to occur in industrial practice, and in this way to be an effective means for enhancing the reliability of industrial-scale nonlinear chromatography.

Local-equilibrium theory

Although chromatographic models that account for transport processes are widely used, models where adsorption equilibrium is assumed locally at each position in the column are generally simpler to solve, and often provide a more fundamental view of the column behavior. A recent example of work of this type was reported by Lendero *et al.* [33] who developed a simple method to characterize the ion-exchange capacity of a column packing. Their approach incorporated aspects of the local-equilibrium theory for an inadvertent pH transient developed by Soto Pérez and Frey [34], as well as an earlier computer-aided method for the design of a chromatofocusing process developed by Kang and Frey [35]. Because the method is simple, non-contaminating, and can be used on-line, it is highly suitable for quality control and in-process monitoring in a current good manufacturing practice (cGMP) environment.

Affinity chromatography

Affinity chromatography is almost always preferred as an early step in a protein purification sequence, as its specificity can greatly facilitate subsequent purification steps. Although affinity chromatography is a well-established method, in the past few years significant progress has been made to further increase its utility.

Inteins

The use of protein fusion technology makes it possible to apply affinity chromatography to essentially arbitrary proteins by fusing the target protein to an affinity tag so that the product can be selectively bound to a column packing functionalized with an appropriate ligand [36•,37]. Although this approach is promising for many applications, its use requires additional separation steps to remove not only the cleaved affinity tag, but also to remove the protease employed to cleave the tag and any nonspecifically cleaved proteins.

Recently, many of the technical problems associated with fusion-based affinity chromatography have been addressed through the use of self-cleaving affinity tags that incorporate 'inteins' (INTervening protEIN sequences), which are naturally occurring protein

elements that exist as in-frame insertions in a host protein. By using an intein to link a target protein to an affinity tag, and by taking advantage of a variety of cleaving strategies, an elution step can be performed where essentially only the target protein appears in the eluate (see Figure 3) [38–40]. For example, in the IMPACT-CN system available from New England Biolabs (Beverly, MA), the target protein is fused to the *Saccharomyces cerevisiae* VMA1 intein with a chitin-binding tag, and self-cleavage is induced using a thiol [41]. To further improve the basic method, Wood *et al.* [42] recently developed a mini-intein that displays rapid C-terminal cleavage when the pH is changed, so that the use of thiols can be eliminated along with their associated cost, toxicity and denaturing properties. Other recent progress has included investigations into the use of inteins on a microfluidics platform [43•] and the use of intein technology, together with a centrifugation step, in an *Escherichia coli* strain that simultaneously produces polyhydroxybutyrate granules that act as adsorbent particles [44••].

Single-domain antibody fragments

In immunoaffinity chromatography, an antibody or antibody fragment is immobilized on a column packing to bind antigens present in the mobile phase. As shown in Figure 4, the antigen-binding domain (Fab) of a typical antibody is composed of the CH1 and VH domains of the heavy chain and the entire light chain. However, it was discovered recently that in dromedaries and llamas, up to one-half of the natural serum immunoglobulin G consists of heavy-chain antibodies (HCAb), which lack the light chain and the CH1 domain [45,46,47•]. In this type of antibody, the antigen-binding domain is composed of the single variable domain VHH, which is 15 kDa in size, and therefore much smaller than the various fragments from conventional antibodies.

VHH fragments can be produced by enzymatic cleavage of HCAb, by cloning VHH genes from B cells or from 'single-pot' libraries. These fragments have been shown by Verheesen *et al.* [47•] to have potential advantages over

Figure 3

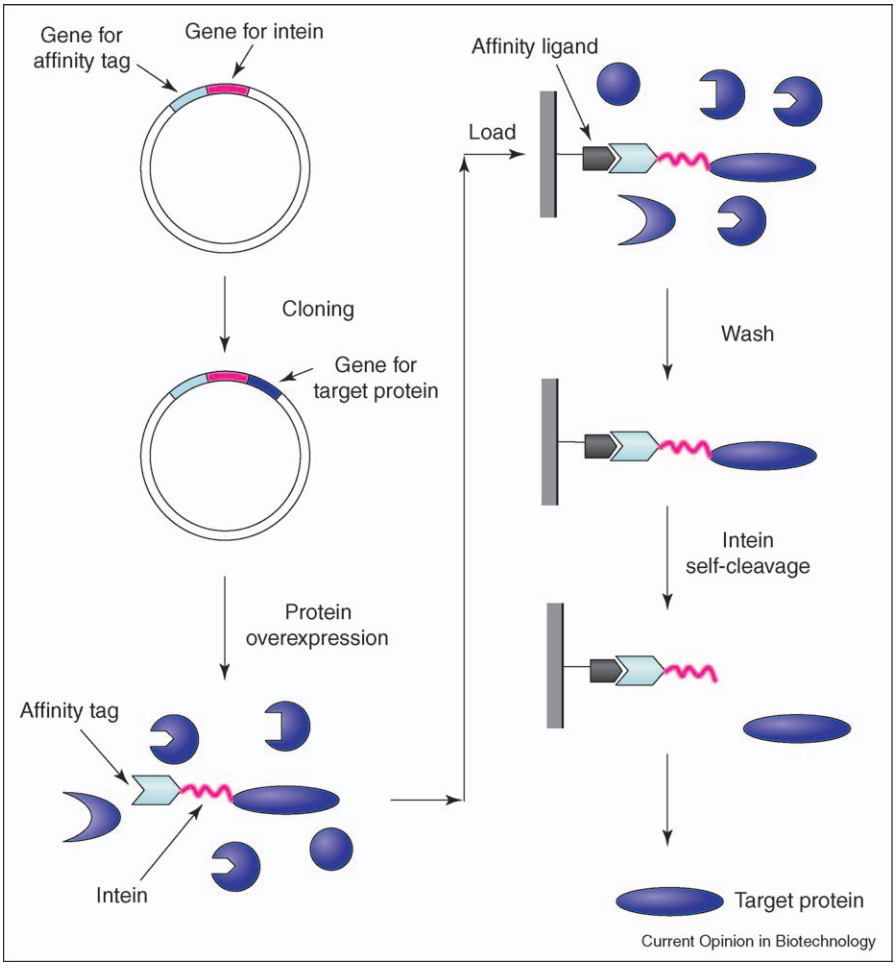
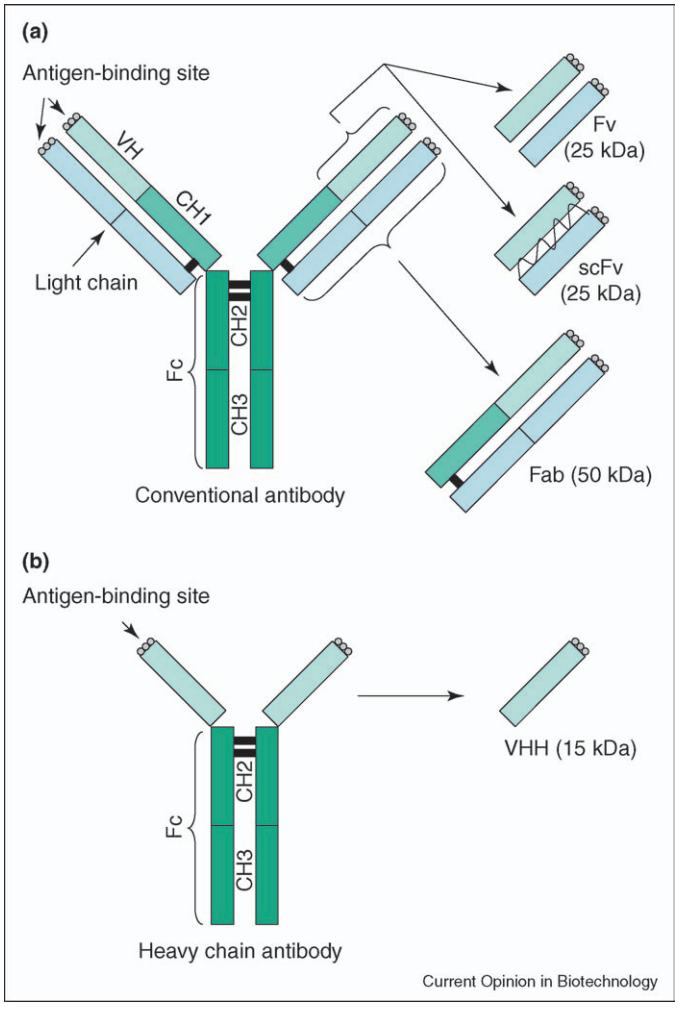


Illustration of an affinity purification method employing an intein linker. The steps shown include cloning, overexpression to produce a tripartite fusion protein, affinity binding, washing, intein self-cleavage, and elution of the target protein.

Figure 4



Structure of **(a)** conventional and **(b)** heavy chain antibodies. Conventional antibody fragments include the antigen-binding fragment (Fab), the variable fragment (Fv), and the single-chain variable fragment (scFv) produced by incorporating a synthetic linker into the Fv fragment. As shown, the VHH fragment is significantly smaller than the fragments from conventional antibodies.

conventional antibody fragments when used in affinity chromatography owing to their high activity to mass ratio, high physical stability, and ability to release bound antigens under mild elution conditions. Recently, the Biotechnology Application Center (Naarden, The Netherlands) has developed and commercialized VHH ligands by using immune llama antibody libraries cloned into an *S. cerevisiae* production strain [48]. Currently, anti-human immunoglobulin G and human serum albumin ligands, and ligands for the purification of human Fab fragments, blood proteins, viral vectors and various tagged proteins and peptides, are available. These are likely to find increased industrial use in the near future.

Synthetic dye ligands

Dye affinity chromatography began as the serendipitous observation that the protein pyruvate kinase binds to the textile dye Cibacron Blue F3GA, which is the dye component of the molecular weight marker blue dextran. This observation quickly led to the study of many similar anthraquinone chlorotriazine dyes for use as ligands in affinity chromatography, owing to their low cost and high chemical stability [49]. Unfortunately, this early generation of dye-based ligands suffered from several shortcomings, such as poor selectivity. Subsequently, a second generation of triazine-based ligands was developed where the various substituents were chosen by considering the structure of natural biological ligands, such as cofactors and inhibitors [50]. Although these more recent ligands performed better than the earlier ones, their potential was still not fully exploited owing to the simplistic approach used in their development. However, as discussed in recent articles [51–54], modern computer molecular modeling tools, especially when used in combination with bioinformatics and combinatorial techniques, now have the potential to produce triazine-based ligands with

binding affinities and selectivities for a target molecule far exceeding previous generations. Consequently, although not an entirely 'new concept', the approach is included in this review, as it now seems poised for significantly increased use in both early-stage capture and late-stage polishing applications. For example, Katsos *et al.* [55] recently developed a triazine-based ligand bearing a ketocarboxylated terminal structure that achieved a 355-fold purification of L-glutamate oxidase with 95% recovery directly from a crude culture broth in a single chromatographic step. Additional recent investigations contributing to the effectiveness of the method, although not specifically concerned with the ligand structure, include the work of Yoshizako *et al.* [56] who studied the use of thermoresponsive polymers to regulate protein binding.

Additional developments in affinity chromatography

Several other recent developments have also increased the effectiveness of affinity chromatography for both small- and large-scale purification. One important example is the introduction by GE Healthcare (Piscataway, NJ) of a variant of Protein A with increased alkaline stability [57]. This variant is incorporated into the MabSelect SuRe column packing for use in Protein A affinity chromatography for antibody purification. The alkaline stability of MabSelect SuRe permits cleaning-in-place with up to 0.5 M NaOH, so that less desirable cleaning reagents such as guanidine HCl can be avoided. As another example, and in keeping with the trend toward using more flexible manufacturing methods discussed earlier, Brorson and co-workers have proposed that bracketed viral clearance studies can be applied to affinity chromatography, as well as to other types of chromatography, in which operating ranges (or 'brackets') of key variables, such as the eluent pH, are identified where acceptable clearance is achieved [58,59]. Revalidation is then unnecessary provided the column is operated within the defined range. One final example is provided by the novel surface coating for silica developed by Wormsbecher [60], which inhibits nonspecific adsorption based on the covalent attachment of hydroxymethyl groups. This technology is employed on the VENTURE line of affinity columns (Grace Vydac, Hesperia, CA), which also utilize rigid, wide-pore silica so that high applied pressures can be tolerated.

Conclusions

Recently introduced concepts in the practice and theory of chromatography are expanding the range of applications for separating peptides and proteins. Future applications of chromatography are expected to take advantage of these new concepts to increase the efficiency of existing applications and to develop entirely new ones.

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