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Review

Expanded-bed chromatography in primary protein purification

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Abstract

Chromatography in stable expanded beds enables proteins to be recovered directly from cultivations of microorganisms or cells and preparations of disrupted cells, without the need for prior removal of suspended solids. The general performance of an expanded bed is comparable to a packed bed owing to reduced mixing of the adsorbent particles in the column. However, optimal operating conditions are more restricted than in a packed bed due to the dependence of bed expansion on the size and density of the adsorbent particles as well as the viscosity and density of the feedstock. The feedstock composition may become the most limiting restriction owing to interactions of adsorbent particles with cell surfaces, DNA and other substances, leading to their aggregation and consequently to bed instabilities and channeling. Despite these difficulties, expanded-bed chromatography has found widespread applications in the large scale purification of proteins from mammalian cell and microbial feedstocks in industrial bioprocessing. The basics and implementation of expanded-bed chromatography, its advantages as well as problems encountered in the use of this technique for the direct extraction of proteins from unclarified feedstocks are addressed.

Keywords: Expanded-bed chromatography; Reviews; Preparative chromatography; Proteins

Contents

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1. Introduction

Production of proteins by genetically engineered microorganisms, yeasts and animal cells became a very important technique for the preparation of pharmaceuticals. The feedstocks from which proteins are prepared are generally complex, containing solid and dissolved biomass of various sizes and molecular masses, respectively. Despite the nature of the starting material, it is necessary to eventually obtain a pure, defined substance of guaranteed purity and potency. This cannot be achieved with a single purification step but is usually achieved with by a combination of different unit operations that account for the different separation necessities, as is shown in Fig. 1.

During cultivation the target protein may be either accumulated in the interior of cells or excreted. Release of accumulated products by the various cell disruption methods yields cell debris of different sizes besides a certain number of intact cells. Whether accumulated or excreted, the first step in a purification protocol is clarification of either the cultivation broth or the cell homogenate. Common methods for the separation of solids and soluble components are microfiltration (MF) and centrifugation (CF). However, both methods show limitations in practice.

In MF, cells and cell debris form a particle layer on top of the membrane surface that not only lead to a decrease of transmembrane flux but also to a partial rejection of proteins. The extend of rejection depends on the molecular mass of proteins, which is due to the ultrafiltration-type characteristic of these dynamic membranes [1,2]. Rejection can be reduced but not circumvented by tangential flow or dynamic filtration

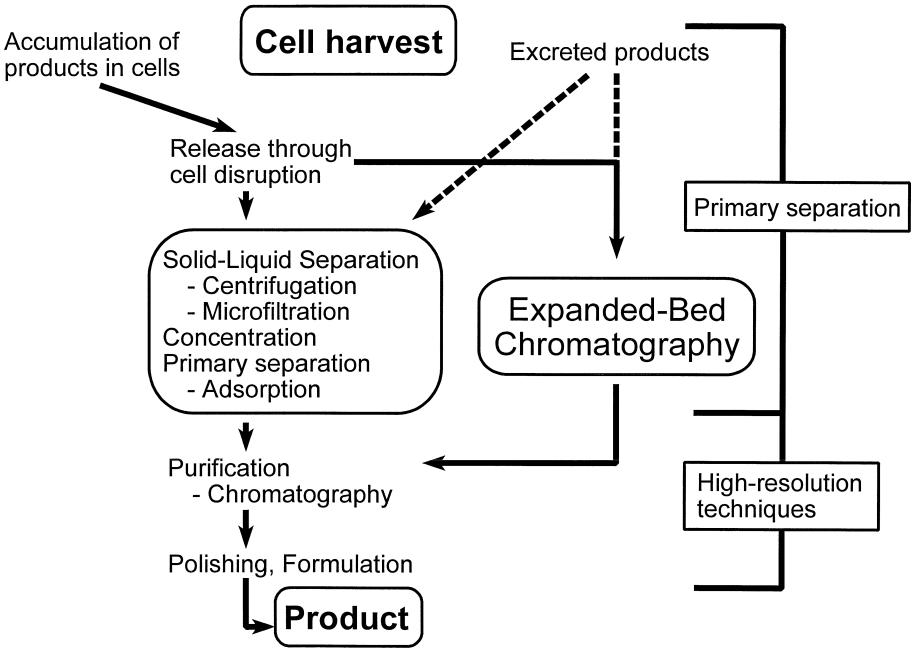


Fig. 1. Process pathways with conventional downstream processes and after inclusion of expanded-bed chromatography.

modules. Furthermore, higher energy inputs are needed and high shearer forces develop, which may affect shear sensitive cells.

In CF, usually continuous centrifuges are employed for large scale processes. With such equipment the removal of biomass is not as effective as with small laboratory centrifuges, why only a reduction of typically two orders of magnitudes is possible. Consequently, CF is often done twice or an additional MF step is included alternatively.

Diluted product feedstocks often require an additional concentration step by ultrafiltration. A primary adsorption step can also be employed for product concentration; here usually an ion-exchange process is employed, which has the advantage to yield partial purification simultaneously.

It must be considered that, besides the cost, the required process time is typically several hours. Therefore, the target protein is in contact with most of the soluble by-products, such as proteases, and is not in a save environment, where oxidation or aggregation can be controlled.

Expanded-bed chromatography allows to integrate solid–liquid separation, volume reduction by protein adsorption and partial purification in one unit operation without compromising on separation efficiencies, but saving considerably processing time and capital investment. The process is based on the use of particulate adsorbents with different selectivities, which reside in a column system. Some reviews are available concentrating on different subjects of this novel technique [3–5].

1.1. Definition of expanded-bed chromatography

The idea of such a whole broth adsorption step is not new. The technique is commonly employed in stirred-tank processes using particles with large particle diameters (0.5–3 mm) and high inner surface area in order to separate adsorbent particles and biomass and to allow for a high capacity for a product. Also column processes are reported, such as for the adsorption of the secondary metabolite streptomycin [6]. Batch processes are composed of several consequent and usually manual operation steps with pouring or pumping the suspensions from one vessel into another in an open environment. As adsorption is not instantaneous, a certain contact time must be designated, so that equilibrium can adjust. If a feedstock is passed through a stirred-tank system, as is shown in Fig. 2, recirculation is recommended to meet residence time requirements. This approach is likely to be inefficient compared with packed-bed procedures, because capture of the target protein is incomplete; adsorption in a stirred tank contributes, at ideal mixing, one equilibrium stage.

However, except for very large beads, a packed-bed column is not suitable for processing feedstocks with suspended biomass, as particles become trapped

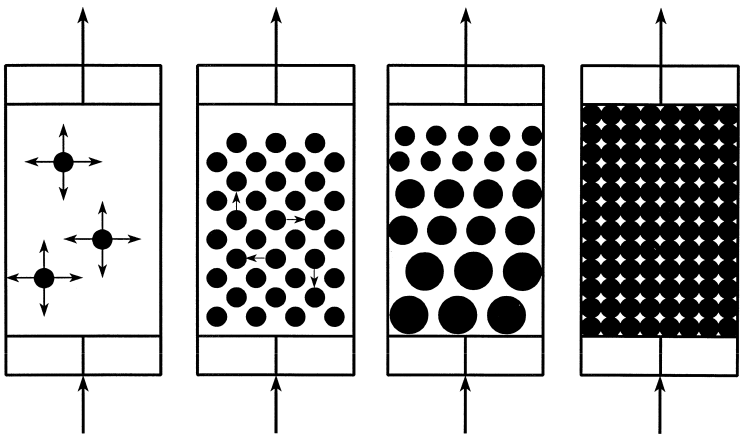


Fig. 2. Operation modes for protein adsorption in column beds with stirred vessel, fluidized bed, expanded bed and packed bed (from left to right).

in the voids of the bed. This results in the formation of a plug of trapped solids near the column inlet and finally to a complete blockage of the column, often after compression of the column bed.

A way out of this dilemma is to pump liquid upwards through a particle bed that is not constrained by an upper column adapter. Then the particle bed begins to expand, if a certain flow-rate is exceeded. Such fluidized beds are known from the heterogeneous catalysis of chemical reactions, where often a gas phase (bubbles) is added to obtain high rates of heat transfer and uniform temperatures within the bed. Mixing in conventional liquid fluidized beds is not as pronounced. However, it is characteristic also for these systems that the adsorbent particles are moving slowly, but constantly in all directions inside the column. Although the performance of a liquid fluidized-bed system is higher compared to the stirred-tank system, it is inferior compared to a packed-bed and may therefore still require recirculation of the feedstock.

Besides rare exceptions, chromatographic particles are not monodisperse and are thus classified in a fluidized bed with the larger particles being located closer to the bottom of the bed and the smaller particles distributed towards the top. If the particles have an appropriate size distribution, classification leads to layers of particles and a reduced mixing in the column. This contributes significantly to the stability of the bed, which then shows fluid dispersion characteristics similar to a packed bed [3]. The appropriate distribution requires a particle size ratio of about greater than 2.2 [7,8]. If also a distribution of the specific density of the particles can be realized, a further stabilization of particle classification is possible. In order to distinguish the dispersion characteristics of classified fluidized beds from those containing particles with relatively narrow size distribution, the term expanded bed was assigned [3,4,9]; expanded-bed adsorption and expanded-bed chromatography are coexisting synonyms.

1.2. Principle of expanded-bed chromatography

After pouring the adsorbent in a column, a random distribution of small and large particles is found in the sedimented bed. In order to achieve the particle size distribution and densities as designated by the manufacturer, complete mixing of the suspension is very important before pouring it into the column, if not the whole content is used. Otherwise, particle size distributions will differ in subsequent column fillings due to classification.

During equilibration the bed is usually expanded by a factor of 2 to 3, thereby increasing the voids between the adsorbent particles and the bed porosity, starting from $\varepsilon_0 \approx 0.4$ in the sedimented bed. After about 30 min the classification within the bed is completed. This can be visually inspected, as then the movement of particles comes to an end.

Subsequently, the feedstock containing cells, cell debris or other particulate matter is applied. As the physical properties of the feedstock are different from those of the buffer used for equilibration — especially the viscosity is higher — the bed further expands if the flow-rate is not decreased. Provided that the settling velocity of the particulates in the feedstock is much lower than that of the adsorbent particles, the particulates leave the column, whereas those proteins are retained that strongly interact with the adsorber.

Then, an intensive washing stage is necessary to remove particulates and weakly adsorbed proteins. It is essential to pass several column volumes ($\approx$20 related to the sedimented bed) of buffer through the column in the expanded mode; otherwise, particulates may remain in the column. Then the flow is stopped, the adsorbent is allowed to settle and finally the upper adapter is lowered. If necessary, further washing can be done in the packed mode in either flow direction.

Usually, elution is carried out in the packed configuration as the product is eluted in a smaller volume. As in packed-bed chromatography, reversal of flow is recommended for step elution. If gradient elution is preferred, the same flow direction as used during adsorption may be more effective. Due to biological safety reasons, movable parts may be unwanted and a closed system preferred. In this case, the upper adapter may be fixed and washing and elution carried out in the expanded mode, the differences being an increased elution time and elution volume.

Some components remain bound under the washing and elution conditions chosen. As the removal of unwanted components follows an exponential de-

cline, a complete removal should not be envisaged. This aspect is particularly important for bacterial cells. A decontamination of the column requires cleaning-in-place (CIP) procedures with reagents such as 1 *M* NaOH and 2 *M* NaCl, up to 70% aqueous ethanol or 6 *M* urea [3] in the expanded mode. Besides the biological hazard that may develop with circumventing CIP, column operation may be severely affected. Channeling may occur due to aggregation of adsorbent particles. Also, the sedimented bed may move upwards as a whole with the next run.

1.3. Columns requirements

The column design must fulfill some basic requirements. The major differences to a packed-bed column are the two adapters. The lower adapter holds a flow distributor — either a plate with holes or a bed of glass ballotini — that ensures a plugged flow in the column by assuring an even pressure drop. This, as a rule of thumb, should be of about the same value as experienced along the column. The distributor enables the passage of particulates without a blockage. An additional screen is placed on top of it; it retains the adsorbent in the column when flow is stopped or reversed.

If the column is to be operated both in expanded and packed mode, altering the position of the upper adapter must be possible. This can be done manually, hydraulically or by using special floating devices. Placing the column in an almost perfect vertical alignment is mandatory. Deviations inevitably lead to inhomogeneous liquid flow and consequently to unstable beds. This is to be specifically emphasized for laboratory-made equipment.

Another column design completely circumvents a lower column adapter; the development of a plug flow is considered irrelevant in the lower part of the column [10]. Here the column is closed at the bottom, but contains laterally connected tubes close to the bottom and a stirrer blade inside. With closing the outlet tube and allowing liquid to flow from the inlet tube to the upper adapter, the bed starts to fluidize. With starting the stirrer, adsorbent mixing takes place in the lower part of the bed, whereas the upper part stays stable with a sharp transition between. Further operation is carried out in the same way as stated above with the difference that elution takes place through the outlet tube. This tube contains a screen in order to retain the adsorbent in the column.The concept reminds of a stirred-tank process at first; however, using adsorbents with appropriate size distribution, classification takes place in the upper part of the column also in this configuration. It remains to be shown whether the mixing and the elution through a laterally connected outlet affects the performance of this system.

2. Theoretical considerations

At very low flow-rate the sedimented bed behaves like a packed bed with the liquid flowing in the interstitial volume of the sedimented bed. Increasing the flow-rate causes a relaxation of the sedimented bed with the minimal fluidization velocity, u_m, characterizing the transition between sedimented and expanded state. At the latter all particles are suspended without having permanent contact to each other. At the relaxation point, the column back pressure is in equilibrium with the force of weight of the adsorbent particles:

$$\Delta P = (1 - \varepsilon)(\rho_p - \rho_l)gh(\varepsilon) \quad (1)$$

Further increasing the flow-rate causes merely an expansion of the particle bed at constant back pressure. Therefore, u_m can be determined from the intersection of the increasing back pressure curve from sedimented bed and constant back pressure in the expanded bed (Fig. 3). Only monodisperse particles show a sharp transition in the back pressure with increasing flow-rate. In practice, relaxation usually takes place over a range of flow-rates owing to the particle size distribution.

The settling velocity of a particle determines the terminal fluidization velocity, u_t. At higher flow-rates a hydraulic transport takes place. In the ideal case of spherical particles the force of weight corrected by the buoyancy and the flow resistance in a liquid are in equilibrium.

$$\frac{\pi}{6} \cdot d_p^3 g(\rho_p - \rho_l) = \xi(\mathrm{Re}_t) \cdot \frac{\pi}{4} \cdot d_p^2 \cdot \frac{\rho_l}{2} \cdot u_t^2 \quad (2)$$

In the range of creeping flow ($\mathrm{Re}_t \leq 0.2$), the resistance coefficient, ξ, is described by Eq. (3).

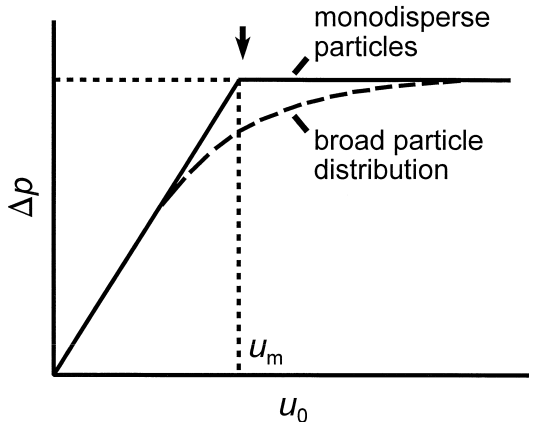


Fig. 3. Dependence of pressure drop and minimal fluidization on the flow-rate of a sedimented particle bed.

$$\xi = \frac{24}{\mathrm{Re}_t} \tag{3}$$

Then the terminal fluidization velocity, u_t, is described by the Stokes' law.

$$u_t = \frac{(\rho_p - \rho_l) d_p^2 g}{18\eta} \tag{4}$$

In this equation the parameters are seen, which play a decisive role in particle fluidization. It is mainly the diameter and density of the adsorber matrix that can be used to control the terminal velocity in the fluidized bed. As the viscosity is an inverse function of the temperature, the bed expansion increases with placing a column from a laboratory to a cold room if the flow-rate is not adjusted correspondingly. Therefore, temperature control is very important to keep control of the fluidization of the column.

To accommodate particulate matter, such as cells or cell debris in the column, the flow-rate during adsorption must be higher than u_m. On the other hand, it must be much lower than u_t in order to keep a manageable height of the column and to prevent a too diluted particle suspension. The useful range of flow-rates is provided by a correlation introduced by Lewis and Bowermann [11]. This is better known as the Richardson and Zaki correlation, as they conducted an extensive investigation of the expansion behavior of various particles at different flow-rates. It is valid for very small particle to column diameters [12]; at ratios $d_p/d_c > 0.01$ wall effects are to be expected [13].

$$\frac{\mathrm{Re}_0}{\mathrm{Re}_t} = \frac{u_0}{u_t} = \varepsilon^n \tag{5}$$

For $\mathrm{Re}_t < 0.2$, which applies to normal expanded bed operations, $n = 4.65$ is constant. However, the expansion index depends both on particle and fluid properties. Furthermore, the most fundamental investigations on fluidization were performed with non-porous particles of uniform spheres [11], high densities and greater diameters as used in expanded-bed chromatography. Consequently, differing values for n were described by Chang and Chase for liquids of different viscosities and porous ion-exchange sorbents [14]. This was also found in this laboratory when n was determined by a least square fitting algorithm according to [15] for liquids of different glycerol content and an ion-exchange sorbent, as shown in Fig. 4. Assuming the volume of the solid-phase does not change with bed expansion, h/h_0 can than be calculated from Eqs. (5) and (6) for an unknown solution with predetermined viscosities (see also Fig. 4). The porosity for the sedimented bed, ε_0, can be measured by application of a large solute that does not penetrate into the adsorbent's pore system. From the random bed theory $\varepsilon_0 = 0.418$ is calculated [16]; as a rule of thumb $\varepsilon_0 = 0.4$ is often assumed.

$$\frac{h}{h_0} = \frac{(1 - \varepsilon_0)}{(1 - \varepsilon)} \tag{6}$$

The expansion behavior can also be estimated from empirical considerations. Hartmann et al. [17] got the best fitting of their experimental data for n using Eq. (7) from Garside and Al-Dibouni [18]; this equation is valid for $10^{-3} < \mathrm{Re}_t < 3 \cdot 10^4$.

$$n = \frac{5.09 + 0.2309\mathrm{Re}_t^{0.877}}{1 + 0.104\mathrm{Re}_t^{0.877}} \tag{7}$$

A more general theoretical view of fluid behavior is based on the Gallileo number [19].

$$\mathrm{Ga} = \frac{\rho_p g(\rho_p - \rho_l) d_p^3}{\eta^2} \tag{8}$$

From Ga the terminal Reynolds number can be

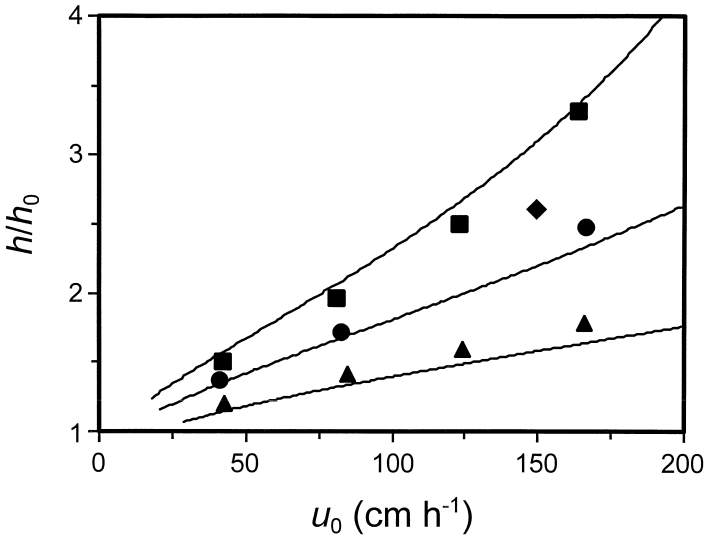


Fig. 4. Relative bed expansion and expansion indices at different flow-rates and viscosities of a newtonian fluid. Streamline SP in 20 m*M* phosphate buffer, pH 7.0, (■) 29.3% glycerol (2.39 mPa s, $n=4.58$, $u_t=1.15$ mm s^{-1}), (●) 20.0% glycerol (1.73 mPa s, $n=5.13$, $u_t=1.94$ mm s^{-1}), (▲) no glycerol added (1.01 mPa s, $n=5.19$, $u_t=3.65$ mm s^{-1}), (♦) *E. coli* homogenate with 2.2 mPa s.

calculated for different column and particle diameters:

$$\mathrm{Re}_t = \left[\frac{23}{\mathrm{Ga}} + \frac{0.6}{\mathrm{Ga}^{0.5}}\right]^{-1}\left[1 + 2.35\cdot\frac{d_p}{d_c}\right]^{-1} \quad (9)$$

from which the terminal flow-rate calculates to $u_t = \mathrm{Re}_t\eta d_p$. Also, the expansion index can be estimated from Ga.

$$\frac{5.1-n}{n-2.4} = 0.016\,\mathrm{Ga}^{0.67} \quad (10)$$

The Reynolds number for minimal fluidization can be estimated according to Riba et al. [20]

$$\mathrm{Re}_m = 1.54\cdot 10^{-2}\mathrm{Ga}^{0.66}Mv^{0.7} \quad (11)$$

with Mv expressed as

$$Mv = \frac{\rho_p - \rho_l}{\rho_l} \quad (12)$$

Tailor-made expanded-bed adsorbents display a broad particle distribution, leading to a gradual transition of bed expansion. Consequently, as long as theoretical models are not available that account for this distribution, the bed expansion characteristic is best obtained by experimental evaluation using solvents of different viscosity, as is shown in Fig. 4.

As the viscosity of microbial homogenates is higher than water and strongly varies with the composition and concentration of the biomass, it cannot be predicted but must be measured. This is usually done by plotting the shear stress against the shear rate, using a rheometer. Only if Newtonian behavior applies or the solution is very diluted, the viscosity can be directly obtained from the slope of this graph. Furthermore, only then the shear rate, $\dot{\gamma}$, can be estimated from the linear liquid velocity, u_0, and the particle diameter, d_p, according to Eq. (13).

$$\dot{\gamma} \approx \frac{u_0}{d_p} \quad (13)$$

However, at high concentrations of a biopolymer solution, non-Newtonian behavior of the fluid is a common observation. This has the consequence that the viscosity is shear rate dependent. A better approximation of the shear rate in an expanded bed column is provided by Eq. (14), considering the flow index, m, and the voidage of the expanded bed, ε [21].

$$\dot{\gamma} = \frac{12u_0(1-\varepsilon)}{\varepsilon^2 d_p} \frac{3m+1}{4m} \quad (14)$$

The flow index is calculated from the power law (Eq. (15)), with τ and K to be obtained from viscosity measurements at different shear rates. From m the shear rate to be considered during expanded bed operation can be estimated. As the shear rate in the expanded bed is very low ($<10\ s^{-1}$), the viscosity is to be extrapolated from the tangential slope of the power law graph at very low shear rates.

$$\tau = K\dot{\gamma}^m \quad (15)$$

The axial mixing within the particle bed is described by the Bodenstein number, Bo. This dimensionless number relates dispersed flow, $D_{ax,l}$, to convective flow ($u_0 h \varepsilon^{-1}$) (Eq. (16)).

$$\mathrm{Bo} = \frac{u_0 h}{\varepsilon D_{ax,l}} \quad (16)$$

At values for Bo>40, axial mixing has little effect on the adsorption performance, i.e. the expanded bed behaves similarly to a packed bed [14]. Bodenstein numbers increase with increasing the height of the sedimented bed. A decrease of the Bodenstein number during operation indicates that the expansion is disturbed by particle aggregation or local turbulences in the column.

2.1. Practical considerations

2.1.1. Matrices for expanded-bed chromatography

Some fundamental work was done with chromatographic adsorbents being used in packed-bed chromatography [3,22,23]. However, besides the relatively unstable fluidized beds that develop with these matrices, their size and specific density are not optimized for a fluidized bed operation. Therefore, operation ranges for agarose or cellulose matrices u_m and u_t are very low, for example $u_t = 222\ cm\ h^{-1}$ for Sepharose Fast Flow, according to Thömmes [5]. This means low flow-rates, long application times and hence low productivities. Denser particles, such as silica, are more appropriate in this respect [24]; however, they are not stable under CIP conditions.

Generally, tailor-made particles for expanded-bed operation are the result of a compromise between the matrix characteristics (particle size, particle density, pore size), which determine the useful range of flow-rates, and adsorption kinetics, especially mass transfer limitations. Very big particles allow high flow-rates according to Eq. (4); however, then very high sedimented bed heights and long columns must be used in order to meet the residence time required for adsorption. Very dense particles, such as zirconia may be used at high flow-rates and can be small in particle size at the same time to reduce mass transfer restrictions. However, small adsorbent particles require a screen with narrow mesh size to retain them in the column, and such screens are prone to be blocked with cells or cell debris.

Tailor-made matrices for protein adsorption in expanded beds are therefore available as hydrophilic polymers made of particulate cellulose, agarose or artificial polymers, such as trisacrylate. These are increased in density by the incorporation of heavier particles, such as quartz, glass, titania or zirconia or metal alloys (Table 1). The sizes of these matrices are generally larger than those of packed-bed adsorbents used for protein purification, and consequently, column performances are low. Expanded-bed chromatography is to be seen as a primary adsorption process, where the target protein — among others — is to be adsorbed in a frontal chromatographic mode. Further purification of adsorbed proteins is not the main intention, though it can be done if it is easily accomplished. The process can therefore also be described as a capture step.

2.1.2. Limitations

As the expanded bed behaves in principle much like a packed bed, mixing in the liquid phase is not the major limiting parameter, as long as some requirements are met. One is the sedimented bed, which must have a minimal height for efficient protein adsorption. Otherwise, dispersion is limiting [Bo<40, see Eq. (16)] and a high percentage of the target protein will be lost in early breakthrough fractions. Also, local turbulences created by the flow distributor are more effective at low bed heights. In practice, the dynamic capacity of an adsorbent is often taken as a measure to compare operation conditions. With the tailor-made adsorbents described above, a minimal sedimented bed height of 100 mm is required for the adsorption of small

Table 1
Properties of particles applicable to expanded-bed chromatography and tailor-made particles

Materials	Particle density (g ml^{-1})	Diameter (μm)	Refs.
Applicable particles			
Fractosil 1000 (silica gel)	1.389	40	[42]
Controlled pore glass	≈1.3	100–250	[43]
Perfluorocarbon	2.2	50–80	[44]
Zirconium dioxide	3.2	50	[45]
Titanium dioxide	2.59[a]	40 and 80	
Tailor-made particles			
Cellulose/titanium dioxide	1.2	125–600	[46]
Agarose/quartz	1.15	100–300	[9]
Agarose/metal alloy	1.3	80–165	[9]
Agarose/glass	1.4–1.6	100–300	[10]
Trisacrylyate/zirconium dioxide	1.5	100–300	[47]

[a] Calculated on the basis of Sachtopore 300 (pore volume 0.12 ml g^{-1}, pore size 30 nm), (Sachtleben Chemie, Duisburg, Germany).

proteins, such as lysozyme on a cation-exchanger [9]. For larger proteins the bed height may need to be increased. It is recommended to start with a sedimented bed height of about 150 mm during method development to avoid the risk of an early breakthrough.

Although low flow-rates and long application times affect feedstock qualities and product stabilities, the flow-rate is limited by the degree of bed expansion. Similar as in the packed-bed mode, the dynamic binding capacity decreases with increasing the flow-rate. However, this is not due to increased axial mixing. Although a general statement regarding the dependence of axial dispersion on flow-rate cannot be given [14], independent investigations showed that the Bodenstein number increases with increasing flow-rate in a range from 100 to 400 cm h^{-1} [25,26], when using tailor-made expanded-bed matrices. It was also shown that a minimal flow velocity in the expanded bed is required to obtain a stable bed [26]. The influence of film diffusion is higher than in the packed bed owing to the increased voids between particles. However, a decrease of the dynamic capacity at increased flow-rates is rather attributed to pore diffusion restrictions [14]. Therefore, the most limiting in expanded-bed chromatography is hindered diffusion of proteins inside the beads, which is also found in most packed-bed adsorption processes.

2.1.3. Composition of feedstocks

The application of a crude feedstock on a chromatographic system is a challenge; however, it can easily get an adventure if the content of the feedstock is not carefully considered. In Table 2 the major components of such feedstocks are compiled. It is only the relative concentration of components that change by moving from a microbial broth to a cell culture suspension. Both are complex mixtures of dissolved components and particulates with the product of varying concentration ratios. Particulates are mainly whole cells with some cell debris in the

Table 2
Composition of a typical product source (broth) after processing by microorganisms or cell cultures

Particulates
Cells (>1 μm)
Cell fragments (<1 μm)
Protein precipitates (<1 μm, colloids)
Dissolved constituents
Low-molecular-mass molecules (M_r<1000)
Amino acids, vitamins, nucleic acids
Buffer salts, additives etc.
Color and aroma substances
High-molecular-mass molecules ($M_r \gg 1000$)
Proteins, including lipoproteins
Polynucleotides (mainly DNA)
Endotoxins

Table 3
Usual characteristics of some cell suspensions

Feedstock	Viscosity (mPa s)	Cell concentration	Refs.
Candida kefyr	2.2	7 g l^{-1} dry mass	[37]
Alkaligenes eutrophus[a]	1.08–1.11	5–10 g l^{-1} dry mass	[23]
Bacillus cereus	1.5–3.0	5–10 g l^{-1} wet mass	[48]
BHK cell culture	1.36	10^7 cells cm^{-3}	[49]
Mammalian cells	1.3		[50]

[a] After cell washing by centrifugation and resuspension in 10 m*M* Tris buffer.

case of animal cell culture and also with microbial bioprocesses if excreted products are to be purified. Besides media components, the feedstock contains mainly the target protein and some other, but minor excreted substances. Especially after cell disruption, many intracellular products, such as host-derived proteins and DNA, are present in large amounts and also high concentrations of endotoxins derived from the cell wall of gram-negative bacteria. Colored by-products are found to different extents. These may adsorb very strongly onto the chromatographic beads and may stay adsorbed under normal elution conditions. This has some similarity with the early days of chromatography, where this ''inner detection'' led to the naming of this technique. Nonetheless, the irreversible adsorption of colored by-products only visualizes what happens also with other feedstock components, such as lipids or endotoxins. Since irreversible adsorption changes the quality of adsorbents with time and is also a problem of contamination, CIP is mandatory in expanded-bed chromatography and is therefore routinely carried out after each run.

2.1.4. Viscosity of feedstocks

During application of the feedstock the bed further expands. A moderate increase does not impair the stability of the bed. However, it can cause the adsorbent beads to pack against the upper adapter if not enough space is left or the flow-rate is not properly adjusted to account for this additional expansion.

The bed expansion is caused by the higher viscosity of feedstocks compared to equilibration buffers, as is shown in Table 3 for different microbial strains and animal cells. Bacterial feedstocks, especially from high-cell-density cultivations with high particle concentrations, display significantly higher viscosities. As this is mainly a result of DNA release by cell lysis, which occurs during growth, cell washing by centrifugation and consequent suspension in an appropriate buffer causes a reduction of the viscosity (Table 3).

A strong effect on the viscosity has long-chain genomic DNA, released by accidental cell lysis or cell disruption. Bacterial homogenates may become very viscous, as is shown in Table 4 for an *E. coli* homogenate. Both dilution and treatment with the enzyme DNase I were necessary before application onto the expanded bed column could be realized [27]. The enzyme DNase I breaks down DNA into small fragments. Samples with very high viscosity cause channeling in the column with a subsequent early breakthrough of the product [28].

Another problem is the correct determination of the viscosity at the low shear rates acting during application on the expanded bed. Since determination by capillary viscosimetry is not possible owing to the particle content, low-shear rheometers should be employed. Rheometers measure shear stress against the applied shear rate (Fig. 5). With Newtonian fluids the viscosity can be taken from the slope of the resulting graph or even at one shear rate. However, as biomass fluids show non-Newtonian behavior, data from a rheometer must be considered more thoroughly. Owing to the pseudoplastic be-

Table 4
Power law (Ostwald) parameters from the rheogram of *E. coli* homogenate (Fig. 5)

Feedstock	Bio wet mass	Consistancy index *K* (Pa)	Flow index *m*	Linear slope, viscosity (mPa s)
Original homogenate	19.3%	0.0914	0.624	40.4
Homogenate with DNase	21.3%	0.0146	0.818	12
DNase and 1:2 dilution	10.7%	0.00383	0.906	2.2

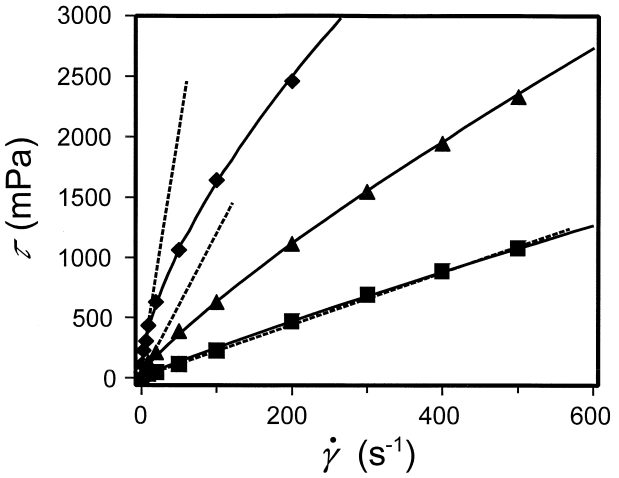


Fig. 5. Non-Newtonian behavior of an *E. coli* homogenate without treatment (♦) as well as after treatment with DNase I (▲) and further 1:2 dilution with water (■). Curves were fitted linearly at low shear rates (- - -) and employing the power law (—) (for parameters see Table 4).

havior of such fluids, the viscosity decreases with increasing shear rates. It would be underestimated if measurements would be restricted to one shear rate, the expansion of the bed higher than expected the consequence. Therefore, the viscosity must be taken from the tangential slope of the graph at shear rates that are to be expected in expanded-bed chromatography.

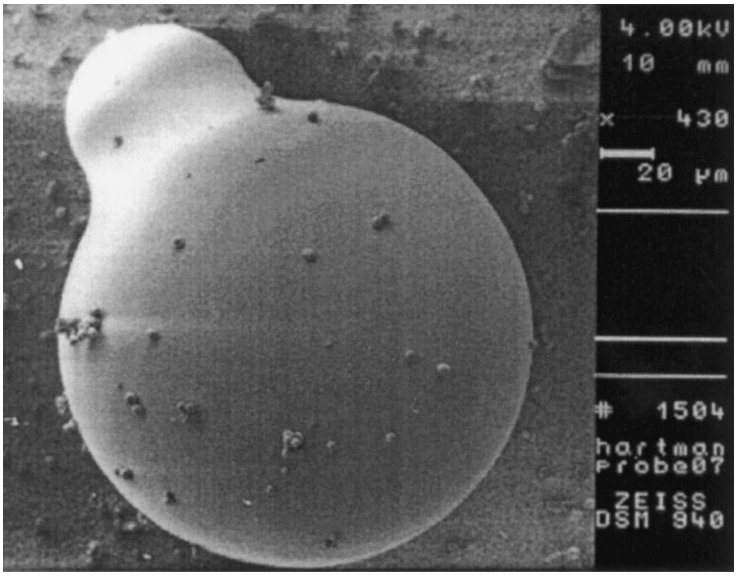
Due to the problems with DNA, avoiding any damage to cells before and during the application of the feedstock is very important if not specifically needed for product release. This is especially true for animal cells, which easily lyse due to shear forces or due to osmotic shock generated by a change of the ionic strength or pH.

2.1.5. Influence of cells on column performance

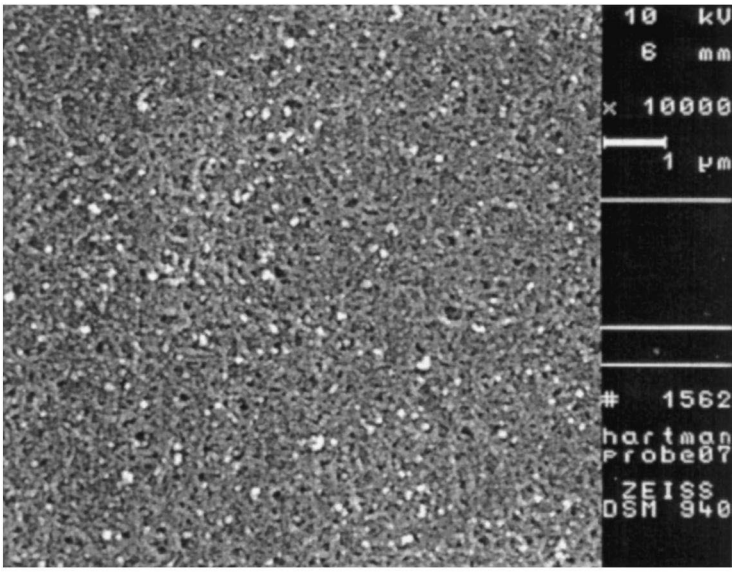
Studies on the influence of cells on the protein binding capacity revealed large effects depending on the selectivity of adsorbents. For example, the BSA binding capacity of a quaternary anion-exchanger dropped about 75% after addition of 2% of *Alkaligenes eutrophus* cells [23]. Also the breakthrough volume was dramatically reduced in the presence of only 0.5% cells to less than 25% of the original value obtained in a cell-free solution. In contrast, a protein A affinity adsorbent yielded only about a 10% decrease in capacity. The same authors found a slight difference between different types of cells, although the general observation did not change [22]. Anion-exchangers display particular problems, which are inherent to their charge. Since the cell wall of bacterial and animal cells are negatively charged, attractive interactions exist to anion-exchangers at the low ionic strengths commonly chosen for protein adsorption. Therefore, competitive interactions at the adsorbents' surface are probably the reason for the decrease in protein capacity observed.

Scanning electron micrographs of the surface of a DEAE anion-exchanger showed adsorbed cells of bakers' yeast after extensive washing of the ion-exchanger with an elution buffer (Fig. 6a). Also remaining cell debris of a homogenate from bakers' yeast is evident on the surface and on pore entrances of this anion-exchanger after extensive washing (Fig. 6b). As during application of crude feedstocks more favorable adsorption conditions are found, the situation will be more severe and competition on the surface with negatively charged proteins can be assumed. Furthermore, as the size of some cell debris fits well with the size of pore entrances, additional mass transfer restrictions will take place at the reduced pore openings.

Also animal cells adsorb at anion-exchangers, as was recently shown by Feuser et al. [29]. In contrast, on a protein A affinity sorbent adsorption was not established, corresponding to the results from



(a)



(b)

Fig. 6. Raster electron micrographs of Streamline DEAE after critical point drying. Adsorption of cells (a) and cell debris (b) of baker's yeast after extensive washing of the anion-exchangers with elution buffer.

Draeger and Chase [23]. However, after long-term application, an apparent loss of cells took place, which was related to cell adhesion at the flow distributor and the lower inlet filter of the expanded-bed column.

These examples demonstrate that scouting in the packed-bed mode and using supernatants is not always to be recommended for the approximation of binding capacities in the expanded-bed mode. One should not be surprised about dramatic changes in

the presence of particulates, and thus process protocols should allow for some flexibility.

2.1.6. Adhesion of cells at the low column adapter

Expanded-bed chromatography has to rely on the difference of particle sizes of adsorber and biomass particulates. Therefore, large cell aggregates are problematic as their hydrodynamic behavior differs largely from a single cell and from cell debris. Cell aggregates can block the screen or the flow distributor at the lower column adapter, causing turbulences in the lower part of the column. If aggregates increase in size inside the column due to incorporation of further cells, these may not be able to leave the column as the terminal velocity increases accordingly. Generally, aggregation of dead cells is most pronounced. Constant stirring of the feedstock is therefore recommended in order to minimize aggregation during application.

Another problem are adhesive cells. Hybridoma cells are problematic as these easily build aggregates through cell–cell interaction; they also stick to any kind of surface. CHO cells are much less problematic in this sense.

Cells and cell aggregates also cause aggregation of adsorber particles in the column, consequently leading to a change in hydrodynamic properties, to channeling and to an increase of axial dispersion or even to a complete breakdown of the expanded bed.

Usually, aggregation of cells and cell debris at the low adapter can be controlled by the CIP procedure. However, cleaning must be done immediately after each run. It is also important to remove as most of the cells as possible during the washing step, to avoid entrapment of cells in the interstitial volume of the particle bed. Problems of bed or distributor clogging due to aggregation or adhesion of cells are most likely to take place after the collapse of the bed. If aggregation at the lower adapter is severe, normal CIP will not reach all parts of the column and the problem will resist. As the strength of chemicals used for cleaning is limited by the chemical resistance of the equipment, dismantling the whole system can be necessary.

Lyse of mammalian cells before application can be prevented by on-line adjustment of pH and ionic strength and by keeping the cell suspension in the cold [30]. Constant stirring is recommended to prevent adhesive cells from building large aggregates. However, partial sedimentation can also be advantegeous to reduce the cell load, as was shown for CHO cells [30]. The addition of glucose is recommended to adjust for the osmolarity, but also here high local concentrations must be avoided.

2.1.7. Separation of cells

Despite the problems described above, the amount of both microorganisms and animal cells can be reduced up to five orders of magnitudes [9,31]. This is much more efficient than centrifugation and may also exceed microfiltration, if large pore sizes are used. However, extensive washing with at least 20 sedimented bed volumes is necessary in the expanded-bed mode. It was also shown that always some particulates stick at the lower adapter that are released with flow inversion [31,32]. Hjorth et al. [9] showed that *E. coli* cells were released with increasing the salt concentration in the washing buffer to 0.5 *M* NaCl, which confirms the sticking of cells to anion-exchange surfaces. Lowering the adapter and inverting the flow generally causes the liberation of a relatively low amount of cells or cell debris that ends with sedimentation of the bed. If particulates still stick at the adsorber particles they are trapped in the voids of the bed and are not found in measurable amounts in the elution fractions.

2.1.8. Examples

A rapidly increasing number of expanded-bed applications is found in literature dealing with the purification of proteins and other biological molecules from microbial and mammalian cell culture [33,34]. Because of the problems of cell interaction with anion-exchange adsorbents, cation-exchange adsorbents are often considered as the better choice. As this is the first purification step, the product ratio is enhanced during this process despite the low performance of expanded-bed columns, the extent depending on the feedstock and the type of the product. Besides many host-derived proteins, DNA can be reduced more than 5 orders of magnitudes on cation-exchange adsorbents and also the high endotoxin concentrations after cell disruption can be reduced at least three orders of magnitudes [27,31,35]. DNA and endotoxins are negatively charged and therefore repelled from cation-ex-

changers. In the case of the strongly positively charged human basic fibroblast growth factor (pI = 9.6), purification and concentration factors of 17 respectively, 5 were obtained using Streamline SP [27].

Despite the problems discussed above (aggregation through bridging of particles by DNA or cell particulates), anion-exchange adsorbents were employed to capture net-negatively charged proteins from unclarified homogenates, such as annexin V [28] and exotoxin A from recombinant *E. coli* [36], extracellular inulinase from *Candida kefyr* [37], or BSA from recombinant *S. cerevisiae* [38]. Even plasmid DNA was purified by a DEAE anion-exchanger from a *E. coli* lysate [39]. Hansson et al. [31] indicate that anion-exchange adsorption of *E. coli* cells is avoided at pH 5.5. However, no particular note about destabilisation of the expanded bed due to adsorbent aggregation is given in the other papers although higher pHs were used. It seems that mainly the concentration of solid biomass and the viscosity is limiting [28]. In order to avoid deterioration of the anion-exchange adsorber due to cell adsorption, an extensive CIP procedure is recommended, involving 0.5 *M* NaOH with 1 *M* NaCl at a contact time of at least 4 h, distilled water, 30% isopropanol and 25% acetic acid [36].

Despite adhesion of cells or clogging of cell aggregates at the lower column adapter, expanded bed chromatography has been successfully applied to animal cell cultures, such as antibodies from hybridoma cell lines using protein A sorbents [26,32] or from CHO cells using a cation-exchanger [30].

A further application can be seen in the separation of soluble from precipitated proteins. This is shown by the purification of recombinant human protein C from the milk of transgenic pigs [40]. Here milk proteins were selectively precipitated by the addition of Zn^{2+} and the suspension applied onto an DEAE expanded-bed adsorber.

2.2. Conclusions

Expanded-bed chromatography is a solid/liquid fluidized-bed technique with reduced mixing of adsorbent particles. It is an integrated method, combining particle separation, product concentration and partial protein purification in one method. Capital expenditure can be saved in those cases where large centrifuges and/or microfiltration units are not needed in cell separation, such as with excreted proteins. Saving of time is an additional advantage, which can amount to several hours in a large-scale process [28,30].

In order to meet the conditions of a stable classified bed and to achieve high flow-rates and productivities, tailor-made adsorbents with high specific density, large particle size and large size distribution ($>$2.2) are required. Special column designs are necessary with a flow distributor in the lower adapter (to establish plug flow) and a movable upper adapter. To avoid turbulences in the column, the introduction of air bubbles must be avoided and a vertical position of the column is mandatory.

Some components of the complex biological system, such as cells, cell debris and DNA, disturb the stability of the classified bed, the extent depending on the functionality of the surface of adsorbents. However, in praxis this reduction in column efficiency is not recognized by many users, although it is proved in basic investigations [22,23,29,41]. It seems that working in the closed system and the other advantages mentioned above predominate this affect. In view of these results the question arises whether a stable expanded bed is indeed a prerequisite for this operation mode or whether a normal fluidized bed showing more pronounced particle mixing would work as well. Here also other column designs developed for this purpose should be taken into a critical consideration. Interactions of all components of the crude feedstock among each other and with adsorbents in the expanded-bed system are very complex. Therefore, a simple answer to this question cannot be given by a sole theoretical consideration, despite the well-known fundamentals of thermodynamics, fluidization and mass transport. Due to the same reason exact predictions of the chromatographic behavior in an expanded-bed system should be avoided. Rather a broad experimental basis from data of complex biological feedstocks should be established from which the major controlling factors of interactions to be extracted. Meanwhile it can be only recommended to try this method and to compare the results with those of known downstream protocols.

3. Symbols

$D_{ax,\,l}$	Axial dispersion coefficient of the liquid
d_c	Column diameter
d_p	Particle diameter
g	gravitational acceleration
Ga	Gallileo number
h	Height of the column bed
h_0	Sedimented bed height of the column bed
K	Consistency index
m	Flow index
n	Expansion index
ΔP	Column back pressure
Re_m	Reynolds number of minimal fluidization
Re_t	Reynolds number of terminal fluidization
Pe	Peclet number
u_0	Superficial velocity
u_m	Minimal fluidization velocity
u_t	Terminal fluidization velocity

3.1. Greek symbols

ε	Porosity of the expanded particle bed
ε_0	Porosity of the settled bed, ≈ 0.4
$\dot{\gamma}$	Shear rate
η	Viscosity
ρ_l	Liquid density
ρ_p	Particle density
ξ	Resistance coefficient
τ	Shear stress

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