Korean J. Chem. Eng., 18(2), 149-158 (2001)

Optimization of Large-Scale Chromatography of Proteins

Jan-Christer Janson†

Center for Surface Biotechnology, Uppsala University, Box 577, SE-751 23 Uppsala, Sweden

Abstract–Protein chromatography is a very complex process based on a combination of thermodynamic, kinetic and mass transport phenomena. By virtue of their complicated and delicate surface structures, the behaviour of proteins on various chromatographic media is not easy to predict. Together with the fact that the majority of the chromatographic media for proteins available today are not very well characterized with regard to their detailed chemical and physical surface structures, protein chromatography still has to be regarded as a predominantly empirical science. I.e. the optimization of separation conditions can only be performed by experiments in the laboratory. The scaling-up is then accomplished primarily by increasing the column diameter. This has been shown work well for column diameters up to at least 1,400 mm. The paper will also deal with the characteristics of the most important protein separation media, silica based, polystyrene based and agarose based, and with how to best optimize the conditions for column productivity, both for adsorption types of chromatography and for gel filtration.

Key words: Protein Chromatography, Large-Scale Chromatography, Gel Media, Optimisation, Gel Filtration, Adsorption Chromatography, Column Productivity, Confocal Microscopy

INTRODUCTION

"There are no scale-up problems but mass transport problems", is an as common as appropriate statement for many chemical engineering processes and holds particularly true for the scaling-up of chromatography of proteins at a time when it is no longer the column flow resistance but the low diffusivity of these macromolecules in the chromatographic gel media which is the most important throughput or productivity limiting factor (Fig. 1).

The chromatographic productivity, is here defined as the amount of adequately purified and collected quantity of protein product recovered per unit chromatographic cycle time and unit column bed volume. The most important productivity parameters are:

1. Protein molecular weight and diffusivity
2. Protein binding kinetics and binding sites
3. Protein solubility
4. Gel particle size and size distribution
5. Matrix porosity and pore distribution
6. Matrix rigidity
7. Column bed height/diameter ratio

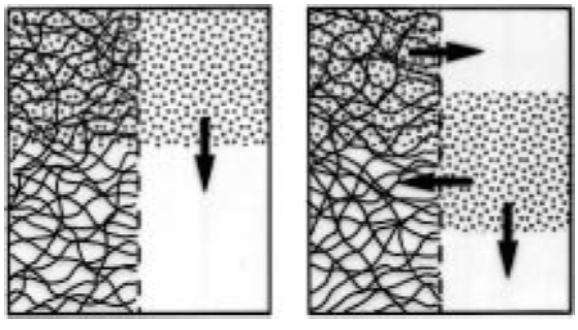

Fig. 1. Schematic representation of the "bottom line" of column productivity in preparative chromatography of proteins in standard industrial gel media. The predominant rate limiting factor is the slow diffusion of proteins in and out of the stagnant liquid in the gel phase.

The parameters at disposal for the biochemical engineer in his effort to improve the productivity of a particular chromatographic process, are very few indeed because of the inherent chemical and physico-chemical characteristics of proteins in general. For any defined ligand and any degree of substitution of that ligand, these parameters are primarily confined to the choice of matrix type, matrix porosity and particle size and to the optimization of column configuration, flow-rate and operating conditions in general.

By virtue of their complicated and delicate, three-dimensional macromolecular structures, developed in a hydrophilic environment, proteins are subject to a number of constraints with regard to the choice of chromatographic column packing materials. An ideal medium for all applications of protein chromatography, would have to meet a formidable number of demands, some of which are contradictory both in theory and practical design. In addition to the factors which govern the performance of protein chromatography columns in general, such as selectivity and efficiency, there are some which are particularly important in an industrial environment, where the final economical balance of the entire process tends to govern the choice of chromatographic medium, with the cost/performance ratio being more important than its pure chromatographic qualities. Two factors that significantly influence throughput are the column flow resistance and the dynamic binding capacity of the chromatographic adsorbent, respectively. The column flow resistance is dependent on the choice of the chromatographic material and its particle size distribution. The dynamic binding capacity, i.e, the binding capacity of the adsorbent for a particular solute under actual working cotions, is a function of several parameters some of which are

†To whom correspondence should be addressed.
E-mail: Jan-Christer.Janson@ytbioteknik.uu.se

inherent with either the solute (protein) or the chromatographic base matrix, some of which are independent, such as the flow-rate applied to the column.

CHROMATOGRAPHIC BASE MATRICES

The chromatographic base matrix or gel, can be characterized as being either a xerogel or an aerogel. Xerogels shrink and swell in the absence and presence of the solvent used, whereas the volume of an aerogel is independent of the solvent. Typical xerogels are cross-linked dextran gels (Sephadex®) and cross-linked polyacrylamide gels (e.g. BioGel P). Typical aerogels are porous glass, silica, and most macroreticular gels based on organic polymers such as polystyrene and polymethacrylate when used in aqueous buffers. In addition to being hydrophilic, an ideal general matrix for protein chromatography should not contain groups which spontaneously bind protein molecules. Rather it should contain neutral functional groups which allow controlled synthesis of a wide variety of selective adsorbents (ion exchangers, HIC adsorbents, RPC materials, biospecific affinity adsorbents, immuno affinity adsorbents etc.). Furthermore, the matrix should be chemically and physically stable in order to withstand extreme conditions during derivatization and maintenance regeneration, CIP (cleaning-in-place), sterilization etc., and be rigid enough to allow high linear flow rates (5 cm/min or more) in columns packed with partiles of diameters down to a few microns. Another important feature of an ideal base matrix is that it should allow the production of gels with a broad range of controllable porosities.

None of the materials mentioned above fulfils all the criteria of an ideal matrix for protein chromatography, they are all compromises. A combination of hydrophilicity with chemical and physical inertness seems best achieved using alcohol hydroxyls or amido groups. This is why the most widely used standard chromatographic media for proteins are based on neutral polysaccharides and polyacrylamide. Of these, cross-linked agarose gels have largely superseeded cellulose as matrices for the synthesis of ion exchangers for protein chromatography. Agarose is also the most important carrier for affinity chromatography ligands. Cross-linked dextran was introduced by Porath and Flodin in 1959 [Porath and Flodin, 1959] and is best known as a gel filtration material (Sephadex®) still widely used in industrial applications for desalting operations using columns of up to 2500 litres volume.

Over the last couple of decades, there has been a trend towards more rigid stationary phases for protein chromatography for two reasons. Firstly, the development of HPLC and FPLC technology for protein separation using 5-10 µm diameter particles and, secondly, the rapid growth of industrial production of recombinant proteins which has given rise to an increased demand for media amenable to packing in industrial scale columns of up to several hundred liters volume.

To meet this demand, there has been a development along two different lines. In the first approach, one has chosen the most rigid material available with adequate porosity and tried to make it compatible with proteins by surface modifications (most of the chemistry of these modifications are proprietory). In this way silica and synthetic organic polymer gels have been designed mainly for HPLC and FPLC applications. In the second approach, well known hydrophilic standard laboratory scale media, e.g. agarose, have been modified by cross-linking procedures for increased rigidity.

SILICA

Porous silica $(SiO_2 \cdot H_2O)_n$ [Iler, 1979] is one of the most important base matrices for the synthesis of HPLC media for protein chromatography. The advantages are the high rigidity and the macroporosity, respectively. In one production process, a sol of sub-micron diameter silica particles is allowed to aggregate into a three-dimensional network, a silica gel. If dried, this network will collapse (shrink) and give rise to a product of little use as a medium for chromatography. However, if the newly formed silica gel is subjected to a process of heat aging, the silica material in the aggregates will slowly rearrange, grow together, to the extent that silica "fibrils" are formed with a concomitant change in curvature of the silica surface from positive to negative (Fig. 2). If the aging process is allowed to proceed further, the gel will eventually disintegrate. In another production process the submicron silica gel particles are mixed with an organic material and spherical aggregates are formed in an emulsi-

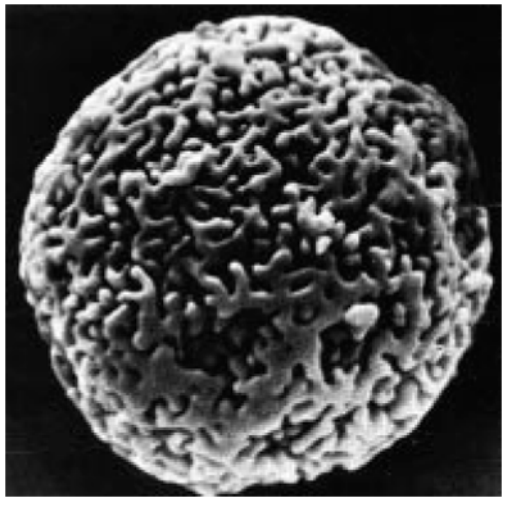

Fig. 2. LiChrospher Si 4000, dp 10 µm (Merck AG, Darmstadt, Germany).

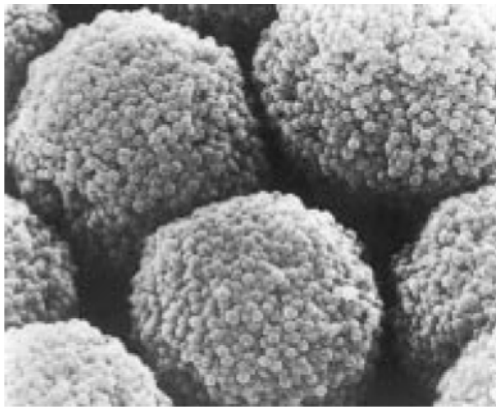

Fig. 3. Zorbax spherical silica gel, 10 µm (Manufactured by Du Pont and Rockland Technologies, Wilmington, Delaware, USA).

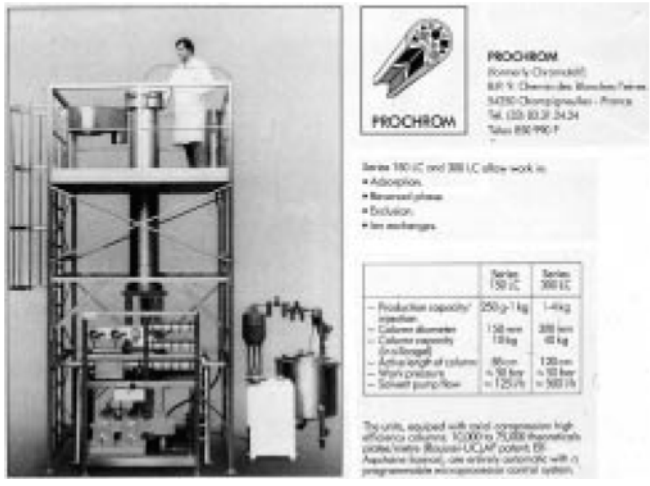

Fig. 4. Industrial scale columns with axial compression used e.g. in the purification of insulin (Courtesy Prochrom).

fication process. The organic material is then removed by heating, a process simultaneously sintering the silica to form stable spherical particles (Fig. 3). The ideal silica surface from a protein chromatographer's point of view, is one containing a high concentration of readily wettable, hydrated silanol groups. These can be functionalized with a number of different silane derivatives followed, if necessary, by end-capping in order to block unreacted silanols which may act as non-specific adsorption sites, or as sites of dissolution attack. The major disad-vantages of silica are the low stability in alkali and the tendency of non-specific, often irreversible, binding of proteins. Nevertheless, 10 μm silica particles are used in columns up to 50 *l* or larger for the final purification of insulin [Kroeff et al., 1989] in the biopharmaceutical industry (Fig. 4).

SYNTHETIC ORGANIC POLYMERS

Synthetic organic polymer based media (resins) have been used for laboratory scale chromatography of peptides and low molecular weight proteins since the early 1950 : es (e.g. Amberlite IRC-50, a methacrylic acid divinylbenzene co-polymer), however, it was not until around 1980 that resins optimized for more general protein chromatography were introduced. These were based on macro-

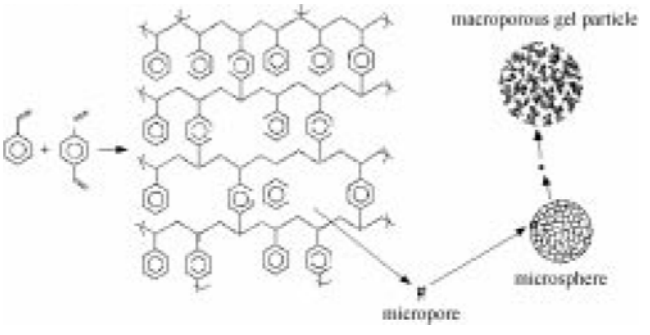

Fig. 5. Schematic representation of the synthesis of a PS/DVB gel particle.

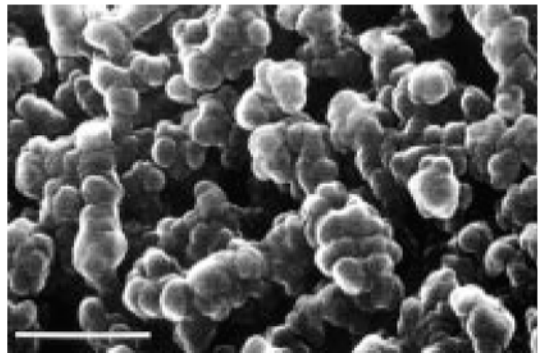

Fig. 6. SEM of the surface of Sephacryl S-200. White bar is 500 nm.

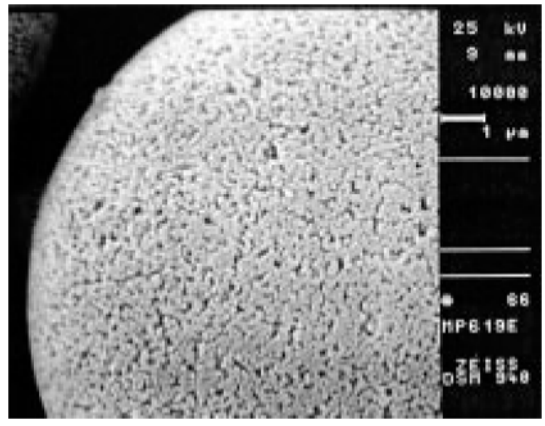

Fig. 7. SEM of the 15 μm PS/DVB media Source 15 (Courtesy Amersham Pharmacia Biotech).

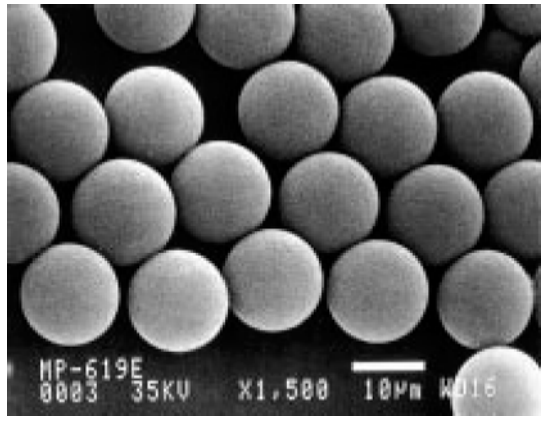

Fig. 8. SEM of the 15 μm PS/DVB media Source 15 (Courtesy: Amersham Pharmacia Biotech).

porous polystyrene/divinylbenzene (PS/DVB) and various acrylic polymers (examples of these are Sephacryl, MonoBeads [Ugelstad et al., 1983], Poros, Toyo Pearl, Spheron and Trisacryl). Fig. 5 shows the schematics of the synthesis of a macroporous PS/DVB gel particle. Figs. 6, 7 and 8 show the surface morphologies of various synthetic macroporous organic polymer media. In Fig. 9 is shown an industrial chromatography system designed for use with 15 μm or 30 μm dp Source™ PS/DVB media in large scale protein purification.

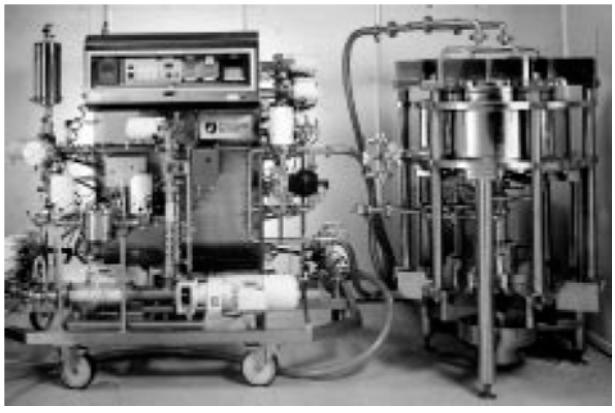

Fig. 9. Industrial scale chromatography system designed for use with 15 µm or 30 µm Source™ PS/DVB media. The volume of the 80 cm diameter stainless steel column is 50 *l*. (Courtesy: Amersham Pharmacia Biotech).

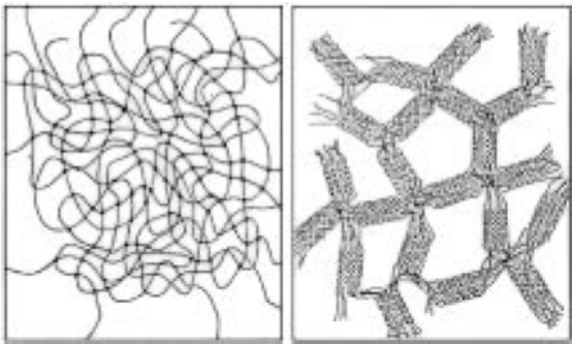

Fig 10. Schematic drawing of a microporous gel type (cross-linked dextran or cross-linked polyacrylamide) to the left and a macroporous gel type (agarose) to the right. From Arnott et al. [Arnott et al., 1974].

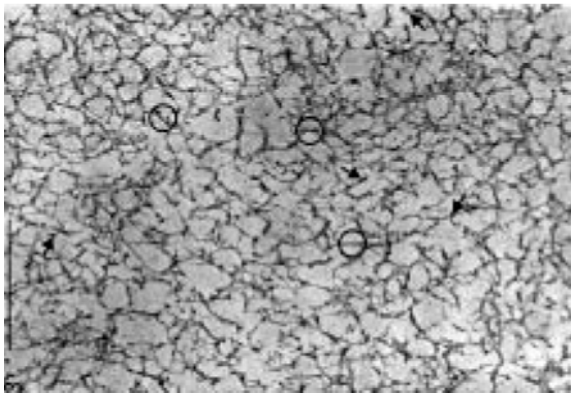

Fig. 11. Transmission electron micrograph of 4% agarose. High quality agarose may form gels at dry weight concentrations as low as 0.4%. However, to be practical as a medium for chromatography, the concentration should be at least 4%. A 4% agarose gel has an exclusion limit for globular proteins of approx. 40,000,000. From Amsterdam et al. [Amsterdam et al., 1975].

CROSS-LINKED AGAROSE

Agarose is a spontaneously gel forming polysaccharide (galactan) originating from red sea weeds. Its neutral hydrophilicity and macroporous gel structure (Figs. 10, 11 and 12) makes it ideally suited for electrophoresis and chromatography of even high molecular weight proteins such as immunoglobulins [Hjerten, 1962, 1964]. Modern agarose gel media for protein chromatography are based on highly cross-linked [Porath et al., 1971, 1975], bead-shaped particles available in mainly three particle size ranges with average particle diameters of 12, 34 and 90 µm, respectively. The 34 µm media are intended for columns with volumes up to a few litres capable of high resolution purification of approximately one kilogram protein per year. The 90 µm media are packed in columns up to several hundred liters volume intended for processing several hundred kilograms of protein per year.

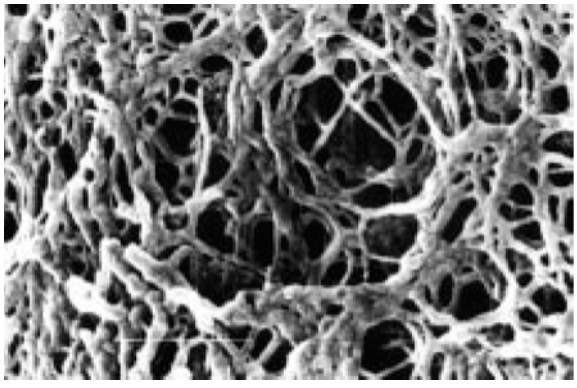

Fig. 12. Scanning electron micrograph of a 2% agarose gel. The thin white bar represents 500 nm (0.5 µm).

COLUMN PRODUCTIVITY PARAMETERS

The basic phenomena which govern the behaviour of proteins in chromatography columns are in principle independent of the scale of operation (Fig. 13). The thermodynamics (equilibria), the kinetics of binding (sorption-desorption) and the mass transport phenomena (convection, diffusion) should execute themselves in the same way in a small column as in a large column, irrespective of column diameter (Fig. 14) provided they are packed to the same particle homogeneity and density, and that the column inlet and outlet devices allow a homogeneous application of the sample feed solution over the entire column cross section; i.e. that the axial pressure drop is radially homogeneous in the column. This can only be achieved when there is practically no radial pressure drop in the

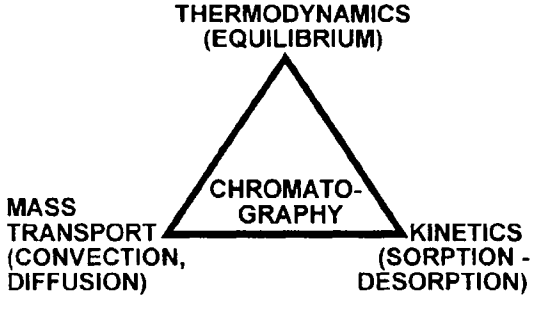

Fig. 13. Chromatography is a very complex process based on thermodynamic, kinetic and mass transport phenomena.

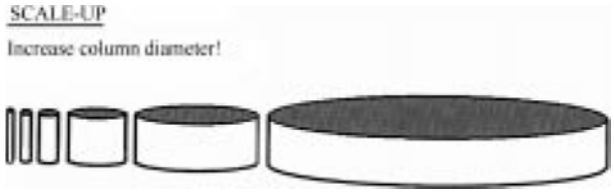

Fig. 14. Scaling-up in chromatography often means increasing the column diameter.

sample distribution layer of the column end pieces. Nor should the volume of this sample distribution layer be too large to avoid excessive dilution of the applied sample zones. The latter requirement, however, is more important for isocratic systems like gel filtration and some reversed phase applications.

The natural first step in any design of a purification process is the selection of a combination of complementary chromatographic chemistries which, using as few steps as possible, gives the desired degree of purification. All optimization of the chemical and physical conditions for each particular chromatographic step, by necessity, takes place in a laboratory scale environment with adequately sized columns, usually with diameters around 1-5 cm. Not only should the chromatographic steps be defined but also their order in the purification process.

The chemical optimization parameters are inherent to each solute/adsorbent combination and thus cannot be discussed in this general context. The physical optimization parameters are, however, common to a wider range of applications and will be discussed in some detail.

One of the most important physical parameters in chromatography is the column length. This is usually optimized in the laboratory and, if the packing density and packing homogeneity, as discussed above, is maintained for the process scale column, then the only required scaling-up factor is the column diameter. If this, for one reason or another, is not possible to achieve, then one might compensate for this by increasing the column length.

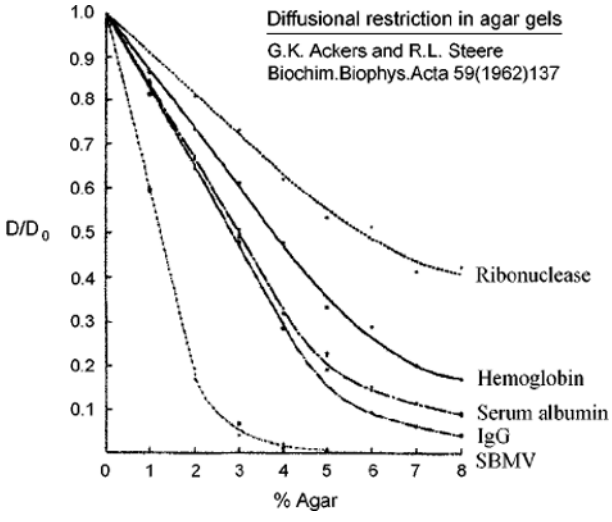

Fig. 15. Ratio of diffusion of proteins in agar gels of increasing concentration (D) to the diffusion in free solution (D_o). SBMV=Southern bean mosaic virus.

$$t_D = \frac{d^2}{2D}$$

For IgG, $D_o = 4.1 \cdot 10^{-7}\ cm^2 \cdot sec^{-1}$

% Agarose		4%	6%
Time (sec) to diffuse	45 μm	82	247
	9 μm	3.3	10

Fig. 16. The "Einstein equation". Average time for a solute with diffusion coefficient "D" to diffuse a distance "d". The relative diffusion of IgG in 4% and 6% agarose is approximately $0.3D_o$ and $0.1D_o$, respectively, as shown in Fig. 15.

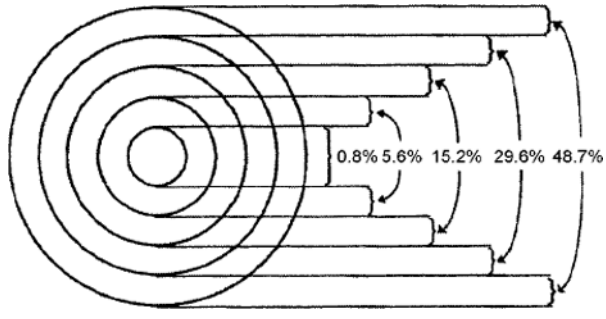

Fig. 17. Schematically, each beaded particle can be thought of as built from a number of concentric shells. The data show % bead volume of each shell.

As has already been mentioned, the major rate limiting parameter in protein chromatography is the restricted pore diffusion in the gel polymer network. In Fig. 15 is shown the relative gel pore diffusion of some proteins in agar gels of increasing concentration (decreasing pore radii) [Ackers and Steere, 1962]. It is obvious that not only the molecular weight but also the shape of the protein molecule governs the relative pore diffusion. It is also clearly illustrated that the lower the molecular weight of the protein to be chromatographed, the higher the applied flow-rate can be with constant resolution.

The "Einstein equation" (Fig. 16) gives an estimate of the average time it takes for a solute to diffuse a distance "d" in a medium with a molecular diffusion coefficient "D". Shown are also the values calculated for IgG in 4% and 6% agarose, respectively. The distances given are related to a spherical agarose gel particle 90 μm in diameter. The value corresponding to a diffusion distance of 9 μm is relevant because it represents a bead penetration of 20% of the radius, a distance which for any particle covers almost 50% of the particle volume and thus almost 50% of the particle binding capacity (Fig. 17). This fact gives an explanation why, in spite of the diffusion restriction for proteins in agarose gels, surprisingly favourable dynamic binding capacities are obtained also for 90 μm diameter particles, even at high linear flow-rates (100-300 cm/h).

Most conditions in process scale adsorption chromatography are based on the assumption of step kinetics, i.e. the rate of protein binding to the ligand, immobilized to the gel matrix back bone, is much faster than the radial diffusion in the particle. This allows the application of the shrinking core model for protein adsorption which is illustrated in Fig. 18. An important restriction in the use of this

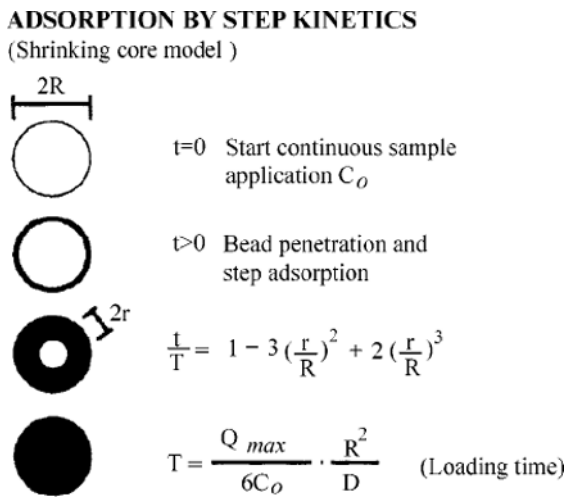

Fig. 18. The shrinking core model, derived from the Einstein equation. T=total loading time, R=particle radius, Q_{max}= maximum loading capacity, r=core radius, C_o=product concentration in feed, D=diffusion coefficient of product in the agarose gel.

simple model is that it requires a constant solute concentration, a condition which is only prevalent at the inlet of the column during sample application.

Recently, using confocal scanning laser microscopy, protein binding to agarose gel based ion exchangers during batch experiments in a finite bath has been studied by Ljunglöf and Thömmes [1998]. "By coupling of a fluorescent dye to the protein molecules the penetration of single adsorbent particles at different times during the batch uptake could be observed visually. Intensity profiles of the protein distribution within a single particle were obtained by horizontal scanning. By integration of the profiles a relative protein concentration within a bead could be calculated. By relating the relative profiles to the intensity distribution at equilibrium, the degree of equilibrium versus time could be constructed. Thus, the batch uptake profile was available from a direct measurement within an adsorbent, and the determination of a transport parameter (effective particle side diffusion coefficient) became possible. These data were compared with uptake profiles obtained by measurements of the protein concentration in the supernatant. The results show that the internal uptake can be measured by confocal microscopy and that the data obtained nicely fit the data from concentration measurements in the supernatant". The data obtained support the concept of the shrinking core model as shown in Fig. 19.

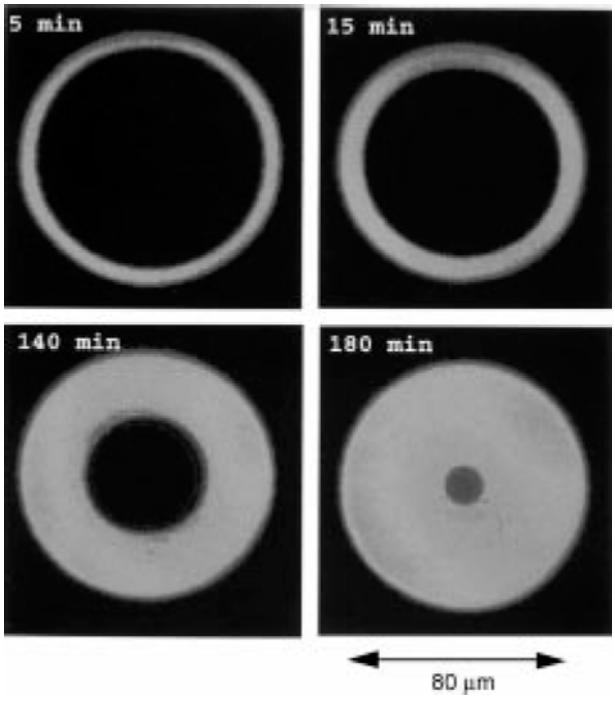

Fig. 19. Confocal images of samples from finite bath uptake of hIgG to SP Sepharose Fast Flow. The times indicate the duration of the incubation [Courtesy Ljunglöf and Thömmes, 1998].

The authors of this paper have continued their studies of this technique and more papers have been published [Ljunglöf and Thömmes, 1998; Linden et al., 1999; Ljunglöf, 1999], not only on protein adsorption but also on the determination of ligand distribution in affinity chromatography media.

A GENERAL STRATEGY FOR THE OPTIMIZATION OF PRODUCTIVITY IN ADSORPTION CHROMATOGRAPHY OF PROTEINS

There are in principle two different modes of operation with respect to utilizing the binding capacity of an adsorbent packed to a fixed bed column. One is to allow the feed stock solution to recycle through the bed at the highest possible flow-rate until the column is saturated and then wash away non-adsorbed proteins, elute the adsorbed proteins in one or several steps (the last for regeneration if required) and finally reequilibrate the column with starting buffer to prepare it for the next sample application cycle. The other mode is to define a maximum tolerated leakage or break-through point, e.g. 1, 5 or 10% of the concentration of the target protein in the feed stock, at which point the sample application is interrupted and the above mentioned washing, elution and reequilibration cycles are initiated. There are two principal differences between these two modes. In the first, the binding capacity of the adsorbent is utilized to its maximum, however, the recirculation of the feed stock makes both the concentration and the composition of the different proteins in the sample change with time and thus not easy to define from one batch cycle to the next. E.g. if the protein content of the feed stock corresponds to 10 times the binding capacity of the column at disposal, then the last cycle will be significantly different in both composition and concentration. In the second mode, the binding capacity of the column is utilized only to a fraction of its maximum, how much depends on the flow-rate used during sample application. On the other hand, as no recycling of feed stock solution takes place, the latter will remain constant with regard to both composition and concentration from one chromatographic cycle to the next.

In Fig. 20 are shown the conditions for two adsorption columns subject to experiments in one-cycle feed mode. For the upper column the flow-rate is high and the target protein in the sample starts leaking out of the column long before the particles of the column

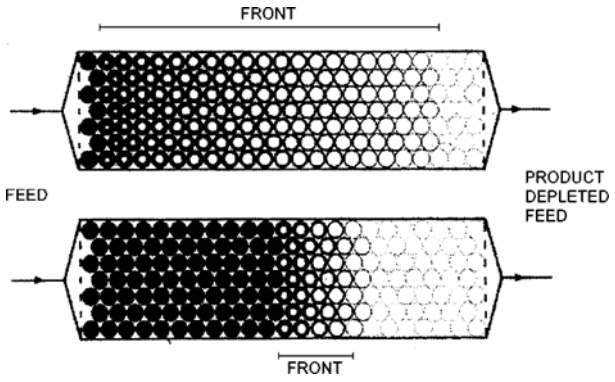

Fig. 20. Sample feeding in one-cycle mode at two different flow-rates.

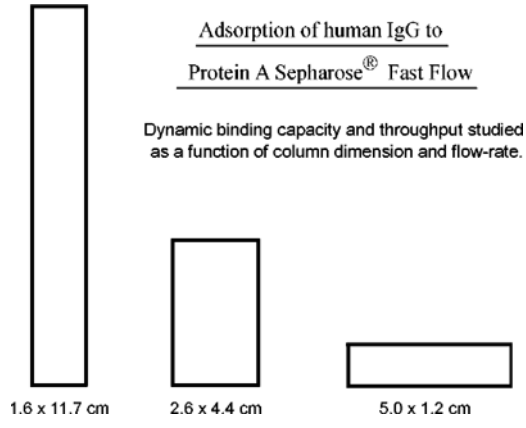

Fig. 21. Three columns of equal volume but different height/diameter ratios used in column productivity studies.

packing are saturated. For the column below, the flow-rate is lower, the resident time of the target molecule is longer and most of the particles in the column will be fully utilized for binding. The question now arises: Which condition will give the highest column productivity? High flow-rate and many cycles or a low flow-rate with few cycles but a higher degree of utilization of the binding capacity of the adsorbent? The answer is that it depends on the ratio of the diameter to the height of the column. To illustrate that this is the case, a series of experiments were made in which the dynamic binding capacity (column loading) and throughput (productivity) for Protein A Sepharose® 4 Fast Flow packed in three columns of equal volume but with different height/diameter ratios (Fig. 21). The sample (1 mg/ml human IgG) was continuously fed to each column at different flow-rates until a break-through of 1% of the inlet concentration was obtained. Then the column was washed, eluted, and reequilibrated and a new sample was applied. All steps of this chromatographic cycle were optimized for each column separately with regard to volume required and flow-rate. The amount of IgG recovered was determined for each column and each flow-rate. The results are shown in Fig. 22.

The longer column (1.6×11.7 cm) shows a higher dynamic binding capacity due to the longer residence time for the IgG in the column. The shortest column (5.0×1.2 cm) shows the highest throughput due to the more efficient mass transfer prerequisites in a column with larger diameter. This is so because at any moment, a much

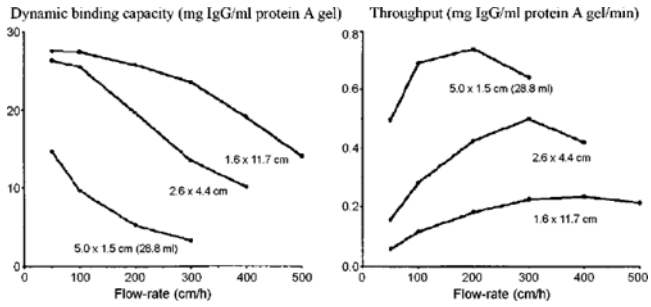

Fig. 22. The effect of flow-rate and column dimension on the dynamic binding capacity (left) and throughput (right) of human IgG to Protein A Sepharose 4 Fast Flow packed in three columns with identical volumes but with different height diameter ratios. Column dimensions same as in Fig. 21.

larger proportion of the total bed volume is in contact with the sample or the washing/equilibration buffer. Even a maximum in the column throughput can be observed at a particular linear flow-rate. This maximum throughput becomes smaller with larger height/diameter ratios. Thus, for any protein sample/adsorbent combination there is an optimum flow-rate for every column diameter/height ratio at constant column volume.

Another important issue to take into account in adsorption chromatography of proteins is the well known effect of residence time on the recovery of active protein. The longer time a protein molecule is bound to a ligand attached to a solid surface, the more chance it gets to change its tertiary structure to fit the chemical topography not only of the ligand, but also of the surrounding matrix surface, and the more points of attachment of different nature that are generated (charge-charge, charge-dipole, hydrogen bonds and bonds based on hydrophobic interaction) the more difficult it will be to elute. The recoveries of both protein and biological activity will decrease and the adsorbent will require more harsher conditions for regeneration. This speaks for shortest possible residence time and thus for the second, one cycle, mode of column operation.

In any industrial purification process there is probably a maximum tolerated cycle time for each chromatographic step and there is often very little to gain, other than the ones discussed above, by reducing this cycle time. This means that the first optimization step in adsorption chromatography of proteins is to adjust the bed height such that it allows a flow-rate, within the pressure rating of the production scale column, that gives the desired cycle time (for binding, washing, elution, regeneration and reequilibration). The desired productivity is then achieved by adjusting (increasing) the column diameter. Remember that at constant column volume, the larger the column cross section, the larger is the proportion of the column packing material that is simultaneously in direct contact with the feed stock solution and thus the better will be the utilization of the chromatographic adsorbent. Also, the shorter the column the higher the linear flow-rate will be at a particular pressure drop applied to this column. And, the higher the linear flow-rate, the shorter time will be required for the implementation of a chromatographic cycle and thus the higher the chromatographic productivity. However, the higher the linear flow-rate and the smaller the column bed height, the lower will the dynamic binding capacity of the column be for a par-

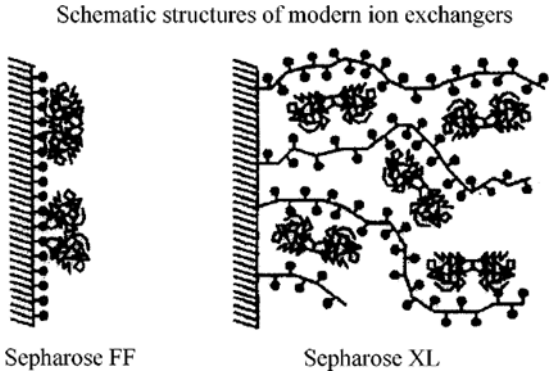

Fig. 23. Schematic drawing of a standard type agarose ion exchanger and a polymer grafted ditto.

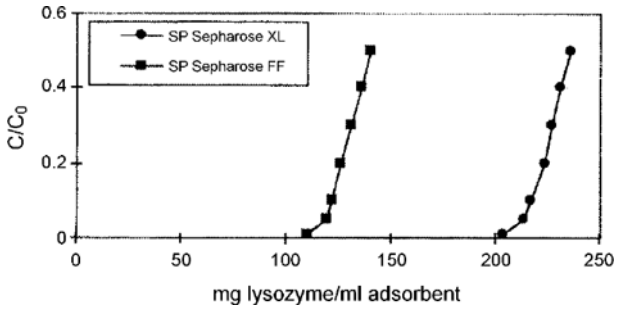

Fig. 24. Break-through curves for lysozyme on a standard agarose gel cation exchanger and on one grafted with a polymer (dextran).

ticular protein. Obviously one has to compromise adsorbent utilization to gain high productivity.

Binding capacity is a crucial parameter in optimization of productivity in adsorption chromatography of proteins. In cases where the binding capacity of standard gel media is insufficient, e.g. for low molecular weight proteins or large peptides, there is now a remedy available. By introducing a polymer "brush" or "tentacle" coating into normal agarose gel media by covalent surface grafting of a hydrophilic, linear polymer such as dextran, two-fold higher binding capacities have been achieved. In Fig. 23 is shown a schematic representation of such a grafted gel medium, in this case an ion exchanger.

In Fig. 24 is shown the result of break-through experiment on a standard ion exchanger and a polymer grafted ditto.

CHROMATOGRAPHIC PRODUCTIVITY IN THE SIZE EXCLUSION CHROMATOGRAPHY (SEC)

The chromatographic productivity in SEC is defined as the amount of adequately purified protein recovered per column cross-sectional area and chromatographic cycle time. The critical parameters in the optimization of SEC productivity are the sample size and flow-rate, respectively.

The sample volume restriction in SEC, as discussed above, makes it mandatory to divide the feed stock into consecutively processed aliquots. The smaller the aliquots, the faster the possible flow-rate at constant resolution. The optimum conditions for processing V_{feed} liters load volume per hour will correspond to [Hagel and Janson, 1992].

$$V_{inj,opt} \simeq (V_{feed} \cdot K_{inj} \cdot V_c \cdot V_p \cdot d_p^2 / 15 D_m)^{1/3}$$

where V_{inj} is the aliquote volume, K_{inj} is related to the shape of the applied sample plug. The optimal value is equal to 12 (the variance of a square-wave distribution) and is approached at larger sample volumes, such as those in desalting operations, but for small sample volumes values around 5 are more common. V_c and V_p are defined as above. D_m is the solute diffusion coefficient in the gel particle and d_p is the average particle diameter. The corresponding linear flow-rate, u, is given by

$$u \simeq V_{feed} \cdot L / V_{inj}$$

where L is the column bed length. The linear flow-rate is obtained by the volumetric flow-rate divided by the column cross-sectional area. These equations are useful for selecting optimum conditions and for scaling-up separation schemes.

Governed by requirements of protein purity and column productivity, it is possible to find an optimum balance between flow-rate and sample volume. One way is to use simulations, correlated with data from experiments, to predict the optimum conditions. Such a study was performed by Arve [Arve] for the separation of IgG and transferrin on Superdex 200. The recovery of IgG at different levels of final purity was determined as a function of flow-rate and sample volume at varying levels of transferrin contamination. Cor-

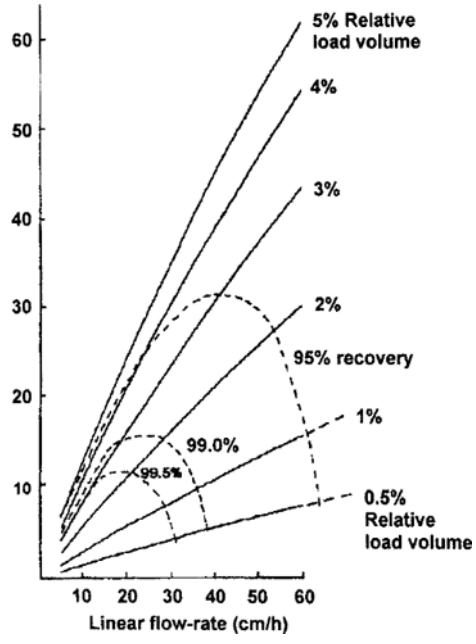

Fig. 25. Computer simulation of productivity and recovery of IgG in GFC on Superdex® 200 prep grade as a function of linear flow-rate and relative sample load (expressed as % of total bed volume). Simulations performed for an initial and final concentration of transferrin of 16.7% and 0.01%, respectively. In order to be able to increase the recovery at maximum productivity, both flow-rate and sample load will have to be decreased. Correlations from experimental work on a 2.6 cm×60 cm column (Courtesy B. Arve).

relations with experimental data were made by computer simulation. The result of one set of conditions with initial and final IgG purities of 83.3% and 99.9%, respectively, are shown in Fig. 25. Of particular interest is the fact that the recovery curves go through a maximum in this operation range. A line drawn through the maxima of these curves should give the combination of sample volume and flow-rate at which one should operate to achieve the desired recovery at maximum productivity. When the optimal conditions for maximum productivity have been established, the cycle capacity is incre ased by proportionally increasing the column diameter and the sample volume.

There seems to be only one way to further increase the column productivity in SEC, and that is to make maximum use of the available separation volume in the operating column, i.e. not allowing any "empty" spaces in the chromatogram. The processing time of an optimized procedure may thus be reduced by 33% by utilizing the dead time during elution of the void fraction to applying a new sample after only 2/3 of the total column volume is eluted. In this way, it is possible in favourable cases to apply three sample cycles per column bed volume. However, this can only be accomplished when the protein of interest and its accompanying impurities are eluted within a rather narrow volume window. Berglöf et al. [1987] described such a favourable situation for the final step in the process purification of human serum albumin. The peaks to be separated were spaced in such a way that three cycles were performed per eluted column volume (Fig. 26) which means that the column productivity could be increased three fold as compared to the traditional way of operating SEC columns. Also, the cycle time can be further minimized my maximizing the flow-rate once the protein of interest has been eluted to rapidly rinse the column before the next sample is applied.

There is a linear relationship between productivity and sample concentration in SEC. However, the restriction is related to the effect of increasing protein concentration on the viscosity of the sample, relative to the eluent, rather than the dry weight content as such. A relatively viscous sample zone is hydrodynamically unstable and viscous fingering soon develops with catastrophic effects on the resolution. As a rule of thumb, the concentration of a globular protein in SEC samples should not exceed 70 mg/ml or a relative viscosity of 1.5.

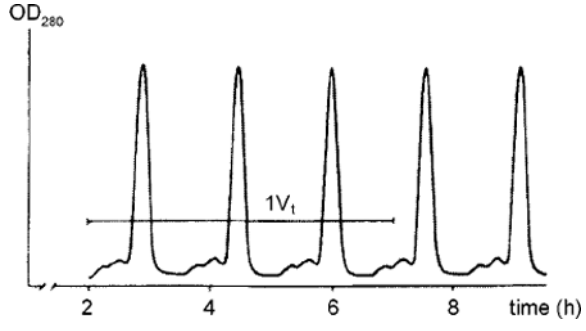

Fig. 26. Optimum utilization of the separation volume in a preparative gel filtration column for the final purification of human serum albumin on Sephacryl S-200 HR. The sample applications are spaced such that three sample cycles are achieved in one column volume, as marked Berglöf et al. [1987].

The most favourable type of SEC from a productivity point of view is desalting or buffer exchange using column packing materials such as Sephadex G-25 which totally excludes proteins but includes low molecular weight impurities and salts. Here sample volumes of approximately 20% of the column volume are routinely used and the flow-rates applied do not have to be adjusted to compensate for the slow diffusion rates of proteins in gels. One way to futher increase the productivity of desalting chromatography unit operations would be to reverse the flow through the column once the target protein has been eluted. In this way slowly moving low-molecular solutes will require a shorter elution distance in order to be removed from the column. At the same time possible particulate material accumulated on the top of the column adaptor filter will be washed out.

STRATEGY FOR SCALING-UP OF SEC

It is known that resolution, R_s, in SEC is more affected by increasing the sample zone width than increasing the operating flow-rate. This is illustrated by a set of experiments where the effect of sample volume and flow-rate on R_s was studied for the separation

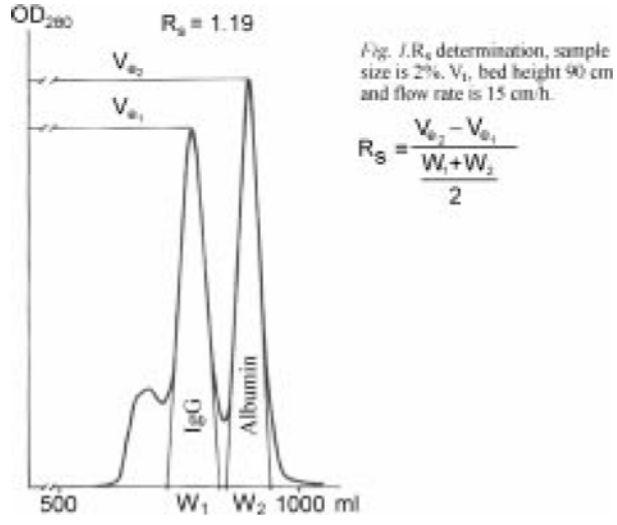

Fig. 27. SEC of IgG and HSA on Sephacryl S-200 HR. Berglöf et al. [1989].

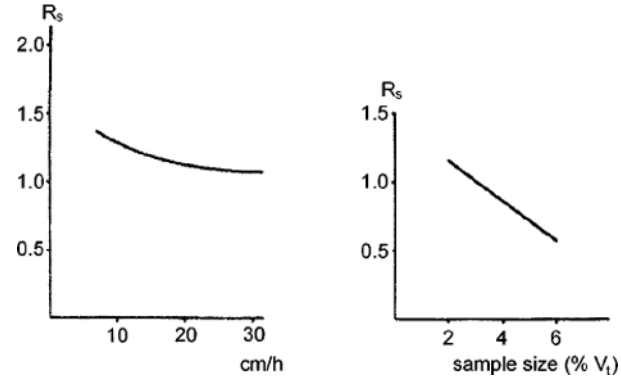

Fig. 28. Effect of flow-rate and sample volume on resolution for the separation of IgG and HSA by SEC on Sephacryl S-200 HR. Berglöf et al. [1989].

of IgG and HSA (see Fig. 27.). The result is shown in Fig. 28. The resolution is obviously much more affected by an increase in sample volume than in flow-rate. This illustration can be used for suggesting a simple strategy for the scaling up and optimization of the column productivity in SEC. Thus, in the first optimization step, one adjusts the column length and flow-rate to obtain a reasonable resolution, but primarily satisfying the desired chromatographic cycle time. In the next step, the desired resolution is obtained by adjusting the relative sample volume. Finally, the desired column productivity is achieved by adjusting the diameter of the gel filtration column keeping the linear flow-rate and the relative sample volume (i.e. the sample zone width) constant. This apparently simple strategy requires that the columns are packed to the same density and homogeneity irrespective of the column diameter. There are many examples in industry that this is possible, but it requires experience and skill in packing large diameter columns. In cases when this is not possible to achieve, one may compensate for less than optimal column packing quality by increasing the column length as discussed by Naveh [1990].

REFERENCES

Ackers, G. K. and Steere, R. L., "Restricted Diffusion of Macromolecules through Agar-gel Membranes," *Biochim. Biophys. Acta*, **59**, 137 (1962).

Amsterdam, A., Er-el, Z. and Shaltiel, S., "Ultrastructure of Beaded Agarose," *Arch. Biochem. Biophys.*, **171**, 673 (1975).

Arnott, S., Fulmer, A., Scott, W. E., Dea, I. C. M., Moorhouse, R. and Rees, D. A., "The Agarose Double Helix and its Function in Agarose Gel Structure," *J. Mol. Biol.*, **90**, 269 (1974).

Arve, B., Pharmacia LKB Biotechnology AB, unpublished data.

As general references on large scale chromatography the following books and chapter, respectively, are recommended reading: Handbook of Process Chromatography, G. Sofer and L. Hagel, Academic Press (1997); Process Chromatography-A Practical Guide, G. Sofer and L.-E. Nyström, Academic Press, 1989; Process Chromatography-A Guide to Validation, G. Sofer and Lars-Erik Nyström, Academic Press, 1991; Large-scale Chromatography of Proteins, J.-C. Janson and P. Hedman, in A. Fiechter (ed.) Advances in Biochemical Engineering, Springer Verlag, **25**, 43 (1982).

Berglöf, J. H. and Eriksson, S., in Biotechnology of Plasma Proteins, Stoltz, J. F. and Rivat, C., (Eds.), Colloque INSERM, **175**, 201 (1989).

Berglöf, J. H., Eriksson, S. and Andersson, I., "Develop. Biol. Standard," S. Karger, ed., Basel, **67**, 25 (1987).

Hagel, L. and Janson, J.-C., in Chromatography, Heftmann, E. (ed.), Elsevier, 5th edition, A267 (1992).

Hjertén, S., "Chromatographic Separation According to Size of Macromolecules and Cell Particles on Columns of Agarose Suspensions," *Arch. Biochem. Biophys.*, **99**, 466 (1962).

Hjertén, S., "The Preparation of Agarose Spheres for Chromatography of Molecules and Particles," *Biochim. Biophys. Acta*, **79**, 393 (1964).

Iler, R. K., "The Chemistry of Silica," Wiley, New York (1979).

Kroeff, E. P., Owens, R. A., Campbell, E. L., Johnson, R. D. and Marks, H. I., "Production Scale Purification of Biosynthetic Human Insulin by Reversed-Phase High-performance Liquid Chromatography," *J. Chromatogr.*, **461**, 45 (1989).

Large-scale chromatography of proteins, J.-C. Janson, and P. Hedman, in A. Fiechter (ed.) Advances in Biochemical Engineering, Springer Verlag, **25**, 43 (1982).

Linden, T., Ljunglöf, A., Kula, M. R. and Thömmes, J., "Visualizing Two Component Protein Diffusion in Porous Adsorbents by Confocal Scanning Laser Microscopy," *Biotechnol. Bioeng.*, **65**, 622 (1999).

Ljunglöf, A., Bergvall, P., Bhikhabhai, R. and Hjorth, R., "Direct Visualisation of Plasmid DNA in Individual Chromatography Adsorbent Particles by Confocal Scanning Laser Microscopy," *J. Chromatogr. A*, **844**, 129 (1999).

Ljunglöf, A., Larsson, M., Knuuttila, K. G. and Lindgren, J., "Measurement of Ligand Distribution in Individual Adsorbent Particles Using Confocal Scanning Laser Microscopy and Confocal Micro-Raman Spectroscopy," *J. Chromatogr. A*, **893**, 235 (2000).

Ljunglöf, A. and Thömmes, J., "Visualising Intraparticle Protein Transport in Porous Adsorbents by Confocal Microscopy," *J. Chromatogr. A*, **813**, 387 (1998).

Naveh, D., "Industrial-scale Downstream Processing of Biotechnology Products," *BioPharm*, **5**, 28 (1990).

Porath, J. and Flodin, P., "Gel Filtration: A Method for Desalting and Group Separation," *Nature*, **183**, 1657 (1959).

Porath, J., Janson, J.-C. and Låås, T., "Agar Derivatives for Chromatography, Electrophoresis and Gel Bound Enzymes. I. Desulphated and Reduced Cross-linked Agar and Agarose in Spherical Bead Form," *J. Chromatogr.*, **60**, 167 (1971).

Porath, J., Låås, T. and Janson, J.-C., "Agar Derivatives for Chromatography, Electrophoresis and Gel-bound Enzymes. III. Rigid Agarose Gels Cross-linked with Divinyl Sulphone (DVS)," *J. Chromatogr.*, **103**, 49 (1975).

Process Chromatography-A Guide to Validation, G. Sofer and Lars-Erik Nyström, Academic Press (1991).

Process Chromatography-A Practical Guide, G. Sofer and L.-E. Nyström, Academic Press (1989).

Ugelstad, J., Söderberg, L., Berge, A. and Bergström, J., "Monodisperse Polymer Particles-A Step Forward for Chromatography," *Nature*, **303**, 95 (1983).