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Optimum support properties for protein separations by high-performance size exclusion chromatography*

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Abstract: Optimum chromatographic properties of high performance size exclusion chromatography (HPSEC) of proteins, such as resolution, molecular weight accuracy and recovery, are obtained on packings and columns with tailor-made physical and chemical structures, employed at properly adjusted eluent compositions and operation conditions. SEC-theory suggests that a broad molecular weight fractionation range and high linearity of the log–linear calibration plot can be achieved by the use of two packings (10- and 80-nm pore size, characterized by a pore-size distribution (psd) equal to or less than 1 decade and by equal internal column porosity ϵ_p), rather than a single 30- to 50-nm pore-size packing with a wide psd. Favourably high phase ratios of $\epsilon_p/\epsilon_o \geqslant 1.0$ for HPSEC columns were accomplished with a minimum interstitial column porosity ϵ_o and a high value for the internal column porosity ϵ_p (the specific pore volume, v_p, multiplied by the packing density, ρ_p.)

Ligands such as diol, *N*-acetoxyamino and oligomeric ether with a propyl- or propoxy-spacer bonded to the silica at the highest density appear to provide high mass recovery and bioactivity as well as chemical stability. Such packings, available in 3–5 μm particle size ranges of narrow distribution, packed into columns >6 mm i.d. and >500 mm in length, offer the best compromise with respect to resolution, speed and pressure drop. More careful studies are required to explain the effects of protein conformational changes and interconversions during elution on HPSEC columns.

Keywords: *Size exclusion chromatography of proteins; properties of size exclusion packings; physical and chemical structure of silica; size exclusion supports.*

Introduction

There are several selective and efficient techniques applicable to the separation of biopolymers [1]. High-performance liquid chromatography (HPLC) offers a wide variety of specific choices for resolving proteins according to their size, charge, hydrophobic character, etc. [1, 2]. Size Exclusion Chromatography (SEC) is a predominantly non-

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interactive elution mode compared to reversed-phase and ion-exchange chromatography. In SEC, resolution is accomplished by selective permeation of the biopolymeric solutes through the internal volume of the column, eluting the proteins in the sequence of decreasing molecular size, in a predictable time interval, and under isocratic conditions. The remarkable separation capabilities achieved in high-performance size exclusion chromatography (HPSEC) of proteins, employing microparticulate hydrophilic bonded silicas and hydrophilic cross-linked organic gels, are well-documented [3–5]. However, full utilization of the separation with regard to resolution, molecular weight accuracy and reproducibility, and mass as well as biological recovery still requires further refinement and improvement of the HPSEC packings themselves.

Focussing on the main goals in HPSEC, the present paper investigates the mutual dependencies between packings and chromatographic properties on the basis of SEC theory. In particular it contains a detailed analysis of the role of physical structure parameters and surface chemistry properties. The extent to which present packings fulfil the predicted requirements and the limits arising in the synthesis of specially designed silica packings are examined. Finally the discussion turns to strategies for running such columns at appropriate eluent compositions and operating conditions, to provide optimum chromatographic resolution and recovery.

Theoretical Background

Prediction of optimum support properties on the basis of SEC theory

In any optimization strategy for HPSEC of proteins there are basically three target features that must be considered: high specific resolution, high molecular weight accuracy and reproducibility, and high mass as well as biological recovery. Favourable values for these quantities are achieved by a wide molecular weight fractionation range, an essentially linear intermediate section of the semi-log calibration curve with minimum slope, minimum standard deviation of the eluted peaks and minimum solute–stationary phase interactions. These characteristics are directly controlled by the physical structure and the surface chemistry of the SEC packing and furthermore require an appropriate adjustment of the operational parameters, such as column length, internal diameter, temperature and the flow rate.

The parameters which define the physical structure of the packing play a decisive role in HPSEC owing to the fact that steric size-exclusion mechanisms determine the elution sequence of proteins. These parameters are: pore size (ps)*, pore-size distribution (psd), specific pore volume (v_p), internal and interstitial column porosities (ϵ_p and ϵ_o, respectively), pore shape, particle shape, particle size (d_p) and particle size distribution (dpsd). Each individual parameter should be weighted according to its effect on the chromatographic data, and this must precede any determination of its optimum value.

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* Abbreviations used in text: ps, pore size; psd, pore-size distribution; vp, specific pore volume; ϵ_p, internal column porosity; ϵ_o, interstitial column porosity; d_p, particle size; dpsd, particle size distribution; v_e, elution volume; [η], intrinsic viscosity; $\bar{a}$, effective pore size; *pr*, mean pore radius; *pd*, mean pore diameter; pr_{50}, value of *pr* at 50% of the cumulative pore-volume distribution; pd_{50}, value of *pd* at 50% of the cumulative pore-volume distribution; pr_m, value of *pr* at the maximum of the relative pore-volume distribution; pd_m, value of *pd* at the maximum of the relative pore-volume distribution; *M*, molecular weight; vpr, ratio of macropore to mesopore volume; R_{sp}, specific resolution; σ, standard deviation of an eluting peak; ρ_p, packing density of material in column; *h*, reduced plate height; *v*, reduced velocity; *u*, linear flow rate; D_{im}, diffusion coefficient of solute in mobile phase; a_s, specific surface area; d_n, size of pore constrictions.

The pore size of the SEC packing, relative to the molecular size, dictates that part of the fractionation range of the column which corresponds to the linear part of the calibration curve: log M vs the elution volume, v_e, or log $\{[\eta] \cdot M\}$ vs v_e, where $[\eta]$ is the intrinsic viscosity and $\{[\eta] \cdot M\}$ the hydrodynamic volume. In theoretical models of SEC separations the pore size is defined in various ways depending on the pore geometry [6, 7]. A useful definition is the expression of the effective pore size $\bar{a}$ as [8]:

$$\bar{a} = 2 \cdot \frac{\text{specific pore volume}}{\text{specific pore surface area}} . \tag{1}$$

In practice the mean pore radius (*pr*) or mean pore diameter (*pd*) are derived from nitrogen sorption or mercury porosimetry measurements, assuming cylindrically-shaped pores; *pr* or *pd* correspond either to the value at 50% of the cumulative pore-volume distribution (pr_{50}, pd_{50}) or to the maximum of the relative pore-volume distribution (pr_m, pd_m). Both values coincide only in the case of a symmetrical Gaussian distribution, but differ in all other cases. The method of measurement, the calculation procedure and the type of mean should be indicated in any comparison of pore-size data.

Theoretical calculations have shown [9] that a single pore-size packing with no psd is capable of fractionating random-coil polymer solutes over 1.5–2 decades of molecular weight, M. In addition the calibration curve does not exhibit a sharp cut-off at the total exclusion and total permeation ranges. Allowing finite values for the psd of the packing, an increasing width of the log-normal psd extends the fractionation range of random-coil polymer solutes; the slope D_2 of the calibration curve increases simultaneously. By extending the width of the psd from one to two decades of ps, the linearity of the calibration curve is impaired.

In order to achieve a wide fractionation range *and* good linearity of the calibration curve, Yau *et al.* [9] advocated the bimodal psd concept for HPSEC packings. A bimodal column set, having $\Delta(\log \text{ps}) = \log (\text{ps}_2/\text{ps}_1) = 0.9$ and no psd, provides a reasonably linear fit over three decades of M. Taking into account the width of psd of both packings and the ratio of macropore to mesopore volume, *vpr*, a bimodal column set with $\Delta(\log \text{ps}) = 1.2$, psd value = 0.15 and *vpr* = 1.0, produces a highly linear calibration curve extending to four decades in M.

When the results of this approach are collated and applied to a practical concept for HPSEC of proteins, it becomes clear that two packings characterized by a mean pore diameter of 10 nm and 80 nm, respectively, with $\Delta(\log \text{ps}) = 0.9$, with the psd of each packing covering one decade without any overlap, and a *vpr* = 1.0, would offer the best compromise in order to obtain a wide fractionation range together with high linearity; this approach is better than one single packing with a wide psd. It must be emphasized that the molecular weight accuracy also depends on the use of pure standards and on the dispersion of the column. The reproducibility, however, is influenced by the flow rate variation of the HPLC system and by other parameters. Himmel and Squire [10] quoted a reproducibility of 15% (as relative standard deviation) in molecular weight determination of proteins, while Andersson [11] found a relative standard deviation of 1.0–1.2% in molecular weight for size exclusion of dextrans.

In SEC, specific resolution, R_{sp}, is given by:

$$R_{sp} = \frac{0.58}{\bar{\sigma} \cdot D_2} \tag{2}$$

where $\bar{\sigma}$ is the average standard deviation of two eluting peaks and D_2 the slope of the calibration curve [12]. For random coil-type solutes, the limiting value of D_2 corresponds to:

$$\text{limiting } D_2 \simeq \frac{1}{3 \cdot v_p} \tag{3}$$

in the case of a single pore-size packing.

Strictly speaking, the decisive quantity is not the v_p in ml of liquid per unit mass of packing, but the internal column volume or porosity. The internal column porosity ϵ_p relates to v_p as follows:

$$\epsilon_p = v_p \cdot \rho_p \tag{4}$$

where ρ_p is the packing density of the HPSEC support in kg $m^{-3} \cdot 10^3$; v_p is given in $m^3kg^{-1} \cdot 10^{-3}$. The larger ϵ_p is, the smaller will be D_2. The limits of increasing ϵ_p arise for practical reasons and are discussed under "Manufacture and Physical Properties of HPSEC Silica Packing Materials".

Model calculations, assuming different pore shapes and spherical solutes, have shown that pore geometry has only a slight effect on the elution volume, v_e, and the slope D_2 [6, 13]. However, these calculations have not to date been verified experimentally, since supports with graduated pore sizes and defined and regular pore geometry are not available.

On the other hand, D_2 is known to be strongly dependent on the shape of the solute molecule, i.e. on its conformation. The width of the fractionation range in the calibration curve of log M vs v_e and also the slope D_2, decrease drastically in the sequence of sphere-like, coil-like and rod-like molecules [14]. This situation is met in the HPSEC of proteins when native globular proteins and, in a second experiment at a different eluent composition, denatured proteins are eluted on the same HPSEC column.

On comparing the two calibration curves it is noticeable that the denatured proteins fall beneath the native proteins [15]. This is attributed to the fact that the more the conformation is extended, the more the solute will be excluded from the pores. Another aspect meriting comment is the problem of possible conformational changes in the native proteins, occurring during their elution on HPSEC columns and induced by solute–stationary phase interactions (cf. surface chemical aspects, below).

It is universally recognized that the particle size of the support has a major effect on column dispersion, expressed in terms of plate height or plate number or as the standard deviation of the eluted peak σ. The smaller the value of σ, the higher the resolution (cf. equation 2). Compared to low molecular weight solutes, the use of biopolymers with small diffusion coefficients therefore has consequences for any strategy directed towards obtaining low dispersion. This is best understood by applying the Knox equation:
$h = B/v + Av^{1/3} + C \cdot v$ [16].
The log–log plot of the reduced plate height, h, vs the reduced velocity, v, shows a minimum for h at $1 < v < 10$; v is given by:

$$v = \frac{u \cdot d_p}{D_{im}} \tag{5}$$

where μ is the linear flow rate and D_{im} the diffusion coefficient of the solute in the mobile phase. Limiting ν between 1 and 10 and if d_p = 10 μm, the linear flow rates for high molecular weight solutes (having $D_{im} = 1 \cdot 10^{-11} m^2 s^{-1}$) are calculated at 10^{-4} (ν = 1) and 10^{-3} (ν = 10) cm s^{-1}. According to equation (5), at constant ν and D_{im}, the flow rate can be increased by reducing the particle diameter. With polystyrenes as solutes Engelhardt and Ahr [17] showed in a recent study that columns packed with 3-μm HPSEC supports can be operated at a linear velocity of about 0.3 cm s^{-1} at the minimum of the h vs ν plot. Again there are practical limitations in the synthesis of small particles with an adequately narrow size distribution. Furthermore, assuming a particle mean pore size of 80 nm and a psd width of one decade, the largest pores in the psd will have openings of the same order as the interstitial voids between the packed particles (300–3000 nm for 3-μm particles).

For obvious reasons spherical HPSEC supports are preferred to angular types:

(i) 3- to 5-μm spherical particles, particularly with high porosities, offer a superior packing stability with regard to higher flow rates and pressures;

(ii) spherical particles of 3- to 5-μm permit a much more homogeneous and stable column bed;

(iii) for angular particles, manufacture by milling leads to problems with the removal of fines of submicron size adhering to the outer surface.

Although the elution of proteins on HPSEC columns is governed by the steric exclusion mechanism, enthalphic processes are often involved, resulting in noticeable alterations in the expected elution volume for specific proteins, i.e. the solvated polymeric solute approaching the solid interface is capable of undergoing ionic and hydrophobic interactions with the surface sites. Donnan exclusion of charged solutes may also occur. The relative strength of the net interactions, reflected by the changes in v_e, depends on the structure of the protein, the surface chemistry of the packing and on the eluent conditions. For basic proteins, e.g. lysozyme, retention at pH 7 decreases with the ionic strength (μ) of the eluent due to weaker ionic interactions. For acidic proteins, e.g. albumin, v_e rises with increasing μ due to a decrease in ion-exclusion effects [18]. Hydrophobic interactions were observed to increase with μ, which also led to an increase in v_e [19].

Adsorption interactions also affect mass and biological recoveries: irreversible adsorption results in a loss of both mass and biological recovery. In the case of reversible adsorption–desorption, partial or complete unfolding of the native protein may occur, depending on whether a few segments or a major part of the biomolecules are involved in the surface attachment. The extent of conformational changes appears to be dependent on the concentration adsorbed and on the width, i.e. on the curvature of the pores of the packing. Structural changes in the adsorption of macromolecules as a function of molecular weight, mass adsorbed and thickness of adsorbed layer have been treated by Glöckner [20]. After desorption into the eluent the unfolded proteins may refold to the native state or remain unfolded. The rate of such interconversions is dependent on the relaxation times. These kinetic effects were recently discussed by Hearn and Grego [21] on the basis of a three-state equilibrium model applied to the elution of proteins on non-polar *n*-alkyl silicas.

Thermal unfolding processes of proteins may also occur in HPSEC, as evidenced for ribonuclease on a Toyo Soda TSK 3000 SW column (B. L. Karger, personal communication). These phenomena result in poor peak shape and multiple peak occurrence and also influence the recovery. Generally it would appear that, in the

context of the stationary and mobile phase, the retention and resolution of proteins in HPSEC are affected by the mass injected, the residence time in the column (i.e. the flow rate) and the column length and temperature.

For proper use in HPSEC, the silica packings need to be modified by bonding hydrophilic ligands at the surface in a highly dense coverage. The most active silanols are removed by modification, albeit still leaving a high population. In a buffered eluent at pH 6–7, the silanols are deprotonated and act as weak acidic cationic surface sites. One of the problems encountered in modification is shielding of the residual silanols by the bonded ligands. This can be done in three different ways, as indicated schematically in Fig. 1 a–c.

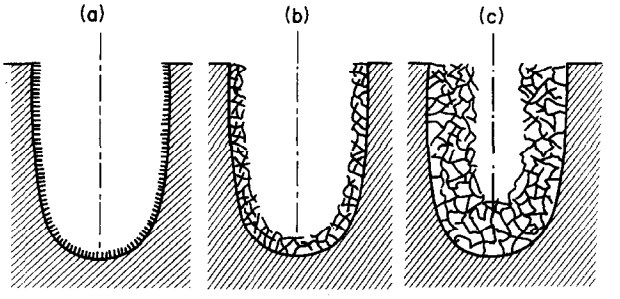

Figure 1
Different forms of bonding structure at the silica surface. (a) Dense monomolecular layer; (b) cross-linked oligomeric layer; (c) polymeric deposit.

Case (a) represents the ideal situation, where a dense monomolecular layer is bonded without any surface defects. In case (b) an oligomeric-type layer is tightly bonded with some cross-linking between the ligands. Figure 1(c) illustrates an extreme case where a thick dense layer of a polymer is deposited at the surface. Type (c) carries two major disadvantages: firstly, the pore volume of the packing decreases proportionally to the layer thickness and this leads to a loss in resolution. Secondly, the layer permeable to solutes impairs the mass transfer kinetics and causes additional column dispersion. Cases (a) and (b) are favoured in terms of fast mass transfer kinetics, provided the ligands are densely packed.

Deactivation of the silica is a major requirement; however, the ligand itself should satisfy certain requirements, too. It should be hydrophilic without any ionic functional groups and should possess a low hydrophobic character. Spacers are usually short-chain *n*-alkyl or ether groups terminated by diol [5], amido groups [22] or *N*-acetylamino groups [23].

Critical evaluation of the HPSEC support properties suggests employment of spherical particles of 3–5 μm with a narrow size distribution. Two support materials of different pore sizes, 10 and 80 nm, each having a psd width of less than one decade with no overlap, are required to match the molecular weight range over four decades and to provide a highly linear calibration plot. A high phase ratio ϵ_p/ϵ_o of >1.0 is favourable to an increase in resolution. Monomeric or oligomeric bonded layers of high density carrying non-ionizable hydrophilic ligands with low hydrophobic character are best suited to minimize elution anomalies.

Manufacture and Physical Properties of HPSEC Silica Packing Materials

The most straightforward concept for the design of a porous solid of defined structure (introduced by Karnaukhov [24, 25]) is to agglomerate non-porous spheres of equal size

(d_p) into compacts of regular array with a distinct coordination number (n). The porosity or specific pore volume is thus a function of n alone, and is independent of d_p. The specific surface area (a_s) is inversely proportional to d_p. The size of pore constrictions (d_n) is equal to the ratio of v_p to a_s or, for silicas, to the product of v_p and d_p [25]. The usefulness of this concept was demonstrated on making compacts from colloidal silica spheres of narrow and unimodal distribution having contact numbers (n) between 8 and 12 [26].

Source and manufacture of packing materials

In the manufacture of porous silica for HPSEC packings, agglomeration is the most frequently used process to control and adjust the physical structure parameters, including particle shape and size. There are several variations employed for the technical implementation, i.e. agglutination, gelling in a water-immiscible liquid, precipitation in a two-phase system and spray-drying (cf. [27]). All these processes yield porous silica beads, mainly with mesopores (mean pore diameter pd_{50} between 3 and 50 nm). Modification of starting materials and operating conditions is required for the synthesis of silicas with macropores (mean pore diameter pd_{50} >50 nm).

For obvious reasons, details on the manufacture of commercial HPSEC silicas according to the above procedures are not always known. In the absence of this information, the discussion is focussed mainly on those properties of commercial silicas which are measurable by appropriate methods. The following silicas are included for comparative studies: the Porous Silica Microspheres (=PSM) product (DuPont de Nemours, Wilmington, USA) made by agglutination of silica sols (No. 1); the spray-dried spherical silica prepared in the authors' laboratories from silica sols (No. 2); two LiChrospher silicas made from tetraethoxysilane (E. Merck, Darmstadt, FRG) (Nos 3 and 4); the angular HPLC silica (Grace GmbH, Worms, FRG) made by a sol-gel process (No. 5); and the silica obtained from a TSK Gel 3000 SW column manufactured by Toyo Soda Company, Tokyo, Japan (spherical silica, manufacturing process unknown) (No. 6).

Silica No. 6 is a hydrophilic-bonded silica well known in the HPSEC of proteins. Silicas Nos 3 and 4 are available in 30 and 50 nm nominal pore size and used as diol-modified derivatives in HPSEC separations. Unmodified silicas of type No. 1 with various pore sizes are widely used in the HPSEC of synthetic polymers. Silicas Nos 2 and 5 are bare silicas which up to now have not been employed in protein separations.

Physical properties of HPSEC packing materials

Figures 2 a–f present the relative pore volume distributions of silicas No. 1–6, obtained by mercury porosimetry using the Washburn equation with 140° as the mercury contact angle and 0.480 Nm^{-1} for the surface tension of mercury at 298 K. Curves are plotted in a log–normal distribution: $\Delta v_p/\log(\Delta pd) \cdot v_p$ vs $\log(\bar{pd})$, and are analysed with respect to pd_{50}, pd_m, the width of psd and the specific pore volume v_p and specific surface a_s according to Rootare and Prenzlow [28].

PSM 500 (*DuPont de Nemours*) (*No.* 1). The psd (Fig. 2a) shows a fairly narrow width of less than one decade of *pd*. The homogeneous distribution is demonstrated by the coincidence between the values for pd_{50} and pd_m: 59.8 and 53.2 nm. The specific pore volume is 0.298 $m^3kg^{-1} \cdot 10^{-3}$ and the specific surface area 19.1 m^2g^{-1}. The packing density ρ_p was found to be 0.76 kg $m^{-3}10^3$ [27].

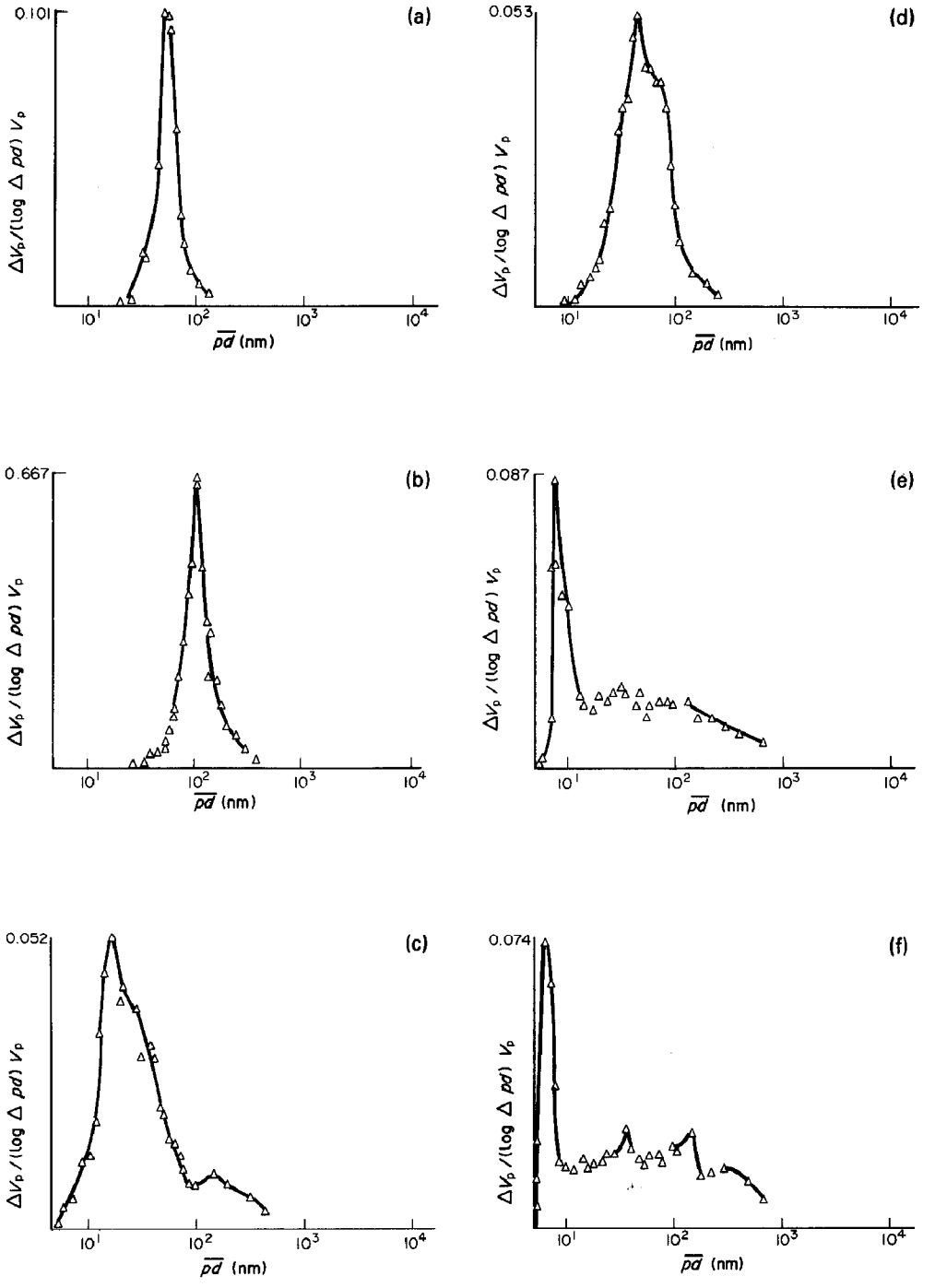

Figure 2
Pore-size distribution of silicas obtained by mercury porosimetry. (a) PSM 500 (DuPont de Nemours, Wilmington, USA; research sample), d_p = 6 μm; (b) spherical silica, made by spray-drying, d_p = 10 μm; (c) LiChrospher Si 300 (E. Merck, Darmstadt, FRG), d_p = 5 μm; (d) LiChrospher Si 500 (E. Merck, Darmstadt, FRG), d_p = 10 μm; (e) angular HPLC silica (250 Å HPLC/10 μm; Grace GmbH, Worms, FRG); (f) packing from a TSK Gel 3000 SW column (Toyo Soda Manufacturing, Tokyo, Japan).

Spray-dried silica produced in the authors' laboratories (*No.* 2). A silica sol of 229 nm mean particle size and a concentration of 12.7% m/m was spray-dried in a Mobil-Minor Spray Dryer (A/S Niro Atomizer, Soeborg, Denmark) at an inlet temperature of 423 K, an outlet temperature of 343 K and a maximum speed of rotation of 35 000 rpm. The psd spanned less than one decade (Fig. 2b). The corresponding pd_{50} and pd_m values were 112.2 and 105.4 nm, indicating a monomodal psd; v_p was 0.250 $m^3kg^{-1} \cdot 10^{-3}$ and a_s was 9.3 m^2g^{-1}.

LiChrospher Si 300 *and* 500 (*E. Merck*) (*Nos* 3 *and* 4). LiChrospher Si 300 packing was 5 μm and Si 500 was 10 μm nominal particle size. The psd of Si 300 spanned more than one decade (cf. Fig. 2c), whereas that of Si 500 spanned about one decade (cf. Fig. 2d); both had distributions less homogeneous than packing Nos. 1 and 2. The pore sizes were respectively: pd_{50} = 30.6 nm and pd_m = 18.8 nm for Si 300; and pd_{50} = 53.8 nm and pd_m = 45.4 nm for Si 500. Other data were as follows: Si 300–v_p = 1.725 $m^3kg^{-1} \cdot 10^{-3}$, a_s = 259 m^2g^{-1}, ρ_p = 0.293 kg $m^{-3} \cdot 10^3$ [27]; Si 500–v_p = 1.105 $m^3kg^{-1} \cdot 10^{-3}$, a_s = 91.5 m^2g^{-1}, ρ_p = 0.373 kg $m^{-3} \cdot 10^3$ [27].

Angular HPLC silica (250 Å *HPLC*/10 μm; *Grace*) (*No.* 5). The psd (Fig. 2e) covers two decades of *pd* and exhibits a heterogeneous distribution with pd_{50} = 38.4 nm and pd_m = 8.1 nm; v_p was 1.421 $m^3kg \cdot 10^{-3}$, a_s = 262 m^2g^{-1} and ρ_p = 0.310 kg $m^{-3} \cdot 10^3$ [27].

Silica packing from TSK Gel 3000 *SW column* (*Toyo Soda*) (*No.* 6). In contrast to the foregoing native silicas, this material was already in the bonded form. The curve in Fig. 2f refers to the original material. The curve for the native silica (after completely burning off the organic content) was also measured but is not shown.

The psd displays the widest distribution so far observed (over more than two decades); the parameters pd_{50} = 44.8 nm and pd_m = 6.4 nm reflect the pore size heterogeneity; v_p = 1.412 $m^3kg^{-1} \cdot 10^{-3}$ and a_s = 290 m^2g^{-1}; ρ_p was not measured.

Some caution is advised in the assessment of the v_p parameter of these highly porous silicas, due to the fracture of particles occasioned by applying high pressures in mercury porosimetry. In the case of the TSK Gel 3000 SW material, fracture was observed at pressures above 400 MPa. Another source of error is introduced by the correction of v_p for the interstitial volume between the particles. In the case of the TSK material the volume of those pores larger than 500 nm (i.e. 0.5 μm) was assigned to the interstitial void. It is assumed that the effective v_p of the TSK packing is higher than 1.412 — probably *ca* 2.0 kg $m^{-3} \cdot 10^{-3}$.

All the aforementioned data characterize the bulk material in its native or modified form. For a critical assessment of the role of physical parameters in HPSEC of proteins the packing stability, the packing density (ρ_p) and the phase ratio ϵ_p/ϵ_o must also be considered. Some of the native silica materials considered above (PSM 500, LiChrospher Si 300 and 500, 250 Å/HPLC/10 μm) were slurry-packed into columns and subjected to stability tests at higher flow rates and pressures [27]. Data were established on the limits of the packing stability with respect to pressure and corresponding flow rates. In summary, microparticulate native silicas with a high specific pore volume, possessing a significant proportion of large macropores (pd > 100 nm), have a pronounced tendency to fracture during the packing procedure.

Where v_p of a packing is given, two additional variables in column manufacturing must

be taken into account, both of which strongly influence resolution in HPSEC through the phase ratio: ϵ_p/ϵ_o. Depending on the packing conditions, both ϵ_p and ϵ_o may be varied independently of each other within certain limits. The internal column porosity ϵ_p is given by

$$\epsilon_p = v_p \cdot \rho_p/m^3\ m^{-3}. \tag{6}$$

As found by Ohmacht and Halász [29] for mesoporous HPLC silicas and by the authors' own investigations on mesoporous and macroporous HPSEC packings [27], a silica with high v_p yields a low packing density ($\sim$0.3 kg $m^{-3} \cdot 10^3$) whereas low pore volume silicas give high packing density ($\sim$0.6 kg $m^{-3} \cdot 10^3$). Ohmacht and Halász [29] also assessed the ϵ_o values corresponding to v_p, ρ_p and ϵ_p. ϵ_o was found to vary between 0.36 and 0.52 for 5 and 10 μm size mesoporous silicas. No correlation was observed between ϵ_o and ρ_p and v_p.

The role ϵ_p plays in the phase ratio of HPSEC packings is illustrated by the data of silicas No. 1 and 3. The internal column porosities ϵ_p calculated according to equation (6) were 0.226 (silica No. 1) and 0.505 (silica No. 3). Multiplying the packing density ρ_p with the specific volume of the truly solid silica $V_s = 0.455\ m^3kg^{-1} \cdot 10^{-3}$ gives ϵ_s, the volume of truly solid silica per m^3 of column volume. ϵ_s values were 0.346 (silica No. 1) and 0.133 (silica No. 3). Then $1-(\epsilon_p + \epsilon_s)$ gives the interstitial column porosity, ϵ_o as 0.428 (silica No. 1) and 0.362 (silica No. 3). The resulting phase ratios ϵ_p/ϵ_o were 0.528 (silica No. 1) and 1.395 (silica No. 3). There is almost a three-fold difference in the phase ratio of the two silica columns while the specific pore volumes differ by a factor of 6.

Given these considerations, high phase ratios are favoured by packings offering a high V_p and a low ρ_p. The price one has to pay is the low packing stability and column stability [27].

It is becoming apparent that none of the commercial HPSEC silica packings supply the optimum support properties required, even when column sets of graduated pore size are employed. Initially a further reduction of the particle size is necessary, while maintaining the packing and column stabilities. This may be critical to some extent when large pore silicas of intermediate v_p and particles <5 μm are employed. Secondly, proper adjustment of the phase ratio should be made by optimizing ϵ_p through v_p and ρ_p. However, determination of a suitable strategy requires further careful experiments. All other properties, such as *pd*, spread of psd, macropore to mesopore volume ratio, etc. can be designed properly during the manufacturing process of the silica.

Surface Chemical Aspects of HPSEC Packing Materials

While the optimum physical structure parameters of packing materials are fairly easy to predict, it is difficult to estimate at this point the most appropriate chemical surface properties with respect to high mass and biological recoveries. Both quantities are specific for any individual protein and very much dependent on the type and strength of adsorption interactions involved. In addition, loss of bioactivity may also arise from mobile phase-induced conformational changes. Elution anomalies as observed for some proteins can be corrected through manipulation of the eluent composition, e.g. type and concentration of electrolyte, addition of modifiers such as amines, methanol and ethyleneglycol.

Through surface modification, i.e. covalent bonding of mono- or multimolecular layers, the silica is deactivated and its surface energy reduced. However, even the modified silica represents an active adsorbent with a high affinity towards proteins. From this point of view, low surface area packings may cause less denaturation and loss in bioactivity of the proteins than high surface area packings. On discussing surface effects and comparing internal surfaces of different columns and column packings, a_s in m^2g^{-1} must be normalized to the unit volume of the column by multiplying it with the packing density, ρ_p. For the PSM 500 silica one obtains: 19.1 $m^2kg^{-1} \cdot 10^3 \times 0.76$ kg $m^{-3} \cdot 10^3 =$ 14.5 $m^2m^{-3} \cdot 10^6$. For the angular HPLC silica (250 Å HPLC/10 μm) the figures are: 262 $m^2kg^{-1} \cdot 10^3 \times 0.31$ kg $m^{-3} \cdot 10^3 = 81.2$ $m^2m^{-3} \cdot 10^6$. Thus the initial a_s-ratio of the two packings (262/19.1 = 13.7) is reduced to 81.2/14.5 = 5.6 by normalization.

The surface area and the pore volume are reduced depending on the mass and volume of the bonded layer. The reduction in pore volume, i.e. in internal column porosity, is more marked because it impairs resolution. The effect is much more pronounced for polymeric deposits than for monomolecular bonded layers, as exemplified for diol-bonded supports [30]. It should be noted that a high organic content (C,H,O) of the modified silica also diminishes the packing density compared to the native form.

Most of the commercial HPSEC silica packings are of the monomeric or oligomeric type and vary considerably in the mass of bonded ligands per unit mass of modified silica. As the ligands often bear ether and/or hydroxyl groups, the carbon content alone is not indicative. For instance, the packing in the TSK Gel 3000 SW column (Toyo Soda) has a carbon content of 7.3% m/m; however, the weight after annealing from 373 to 823 K then amounts to 15.0% m/m. Assuming a bonded mass of 20% m/m with a density of 1000 kg m^{-3} at a surface of $a_s = 200$ m^2g^{-1}, the average thickness of the bonded layer is calculated at 1 nm. This corresponds roughly to the length of an *n*-octylsilyl chain in its extended form, or approximates to the length of a glycidoxypropylsilyl group.

Hence the mass bonded does not serve as an indicator for distinguishing between a monomeric and oligomeric type of ligand. In the case of the monomeric type, the silica is reacted with the appropriate silane and attached to the surface by one or two siloxane links. In oligomeric bonding a reactive monomeric silane is reacted first and, in a second step, treated with a reagent which causes bonding at the first layer and eventually cross-linking between adjacent ligands. Other reaction schemes are also feasible.

In the past a variety of hydrophilic ligands have been bonded to silicas for protein separations (listed in Table 1). Up to the present time there is no definite evidence which type of bonded ligand is best suited with respect to mass recovery, maintenance of bioactivity, chemical column stability and column life-time. A comparison is possible only when the properties of the parent silica, the type of silane and the reaction conditions are known in full. However, detailed information is available on the recovery of proteins on commercial HPSEC packings [31]. In order to assess deviations in the elution of proteins related to pure size exclusion, the retention of specific test solutes, e.g. lysozyme, is measured [31]. Reliable data on column life-time are obtained by flushing several thousand column volumes of eluent through the column and simultaneously monitoring the peak position, peak area and peak height of the test solutes. Such measurements, however, are extremely time-consuming and costly.

In summary, the majority of the commercial HPSEC packings possess acceptable recovery values for most proteins. However, column stability and life-time are found to be insufficient and must be further improved. Reliable strategies for improving the surface chemical properties of packings are not yet available due to the lack of

Table 1
Chemical surface composition of HPSEC silica packings

Structure (bonded ligand)	Type of layer	Reference
$-(CH_2)_n-OH$ ($n = 3,5$)	Monomeric	32
$-(CH_2)_2-CH(OH)-CH_2(OH)$	Monomeric	32
$-(CH_2)_3-O-CH_2-CH(OH)-CH_2(OH)$ (glycerylpropyl)	Monomeric	33
glycerylpropyl	Oligomeric	34
glycerylpropyl	Polymeric	30
$-(CH_2)_3-(CH_2-CH_2-O-)_n-R$ ($n = 1,3$)	Monomeric	35
$-Si(CH_2-CH(OH)-CH_2(OH))_3$	Monomeric	*
$-(CH_2)_3-(CH_2-CH(OH)-CH(OH)-CH-)_n-(OCH_2-CH(O)-CH_2-)_m$ (polyglycydether)	Oligomeric	36
$-(CH_2)_3-NHCOCH_3$	Monomeric	37, 38

* K. K. Unger and A. Sieger, to be published.

meaningful characterization methods for bonded phases and due to the complexity of the system.

Column Dimensions and Operating Conditions

When the support properties are given, the operator has the choice of column length and inner diameter, column temperature, eluent composition and flow rate.

In terms of resolution (cf. equation 2), an increase in column length increases resolution, since D_2 is proportional to the reciprocal of the column length and σ is proportional to the square root of the column length. If 3- to 5-μm size particles are employed, however, the column length can be drastically shortened due to the gain in column dispersion effected by reducing d_p. Short columns of narrow bore, on the other hand, have the disadvantage of losing efficiency through extra-column contributions. This can be counterbalanced by increasing the column's inner diameter, at constant length. In any case, short columns have the distinct advantage of shorter elution times, relative to larger columns at the same flow rate. This aspect comes into play when conformational changes of the proteins occur during elution on an HPSEC column. Flow rates higher than the optimum can be applied, provided the mass transfer kinetics are fast and the pressure drop does not conflict with the viscous heat dissipation. At the present time the available experimental data do not allow the exact conditions to be predicted.

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