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Review

Silica-based supports for high-performance liquid chromatography of biopolymers using non-denaturing conditions

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ABSTRACT

Silica-based supports have been used successfully as media for high-performance liquid chromatography (HPLC) of proteins. Their characteristics of particle diameter, pore diameter, rigidity and suitability for derivatization have made them well accepted for all relevant HPLC techniques. In this article, physical properties and operating parameters of silica-based supports for those HPLC modes which render proteins intact and biologically active will be discussed.

CONTENTS

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1. INTRODUCTION

In the analysis or purification of proteins, and in particular, enzymes, it is usually critical that the native conformation remain intact to retain biological or enzymatic activity. Mild non-denaturing mobile phases and chromatography supports are crucial for success. Until the mid-1970s, all protein chromatography was performed on soft gels which preserved biological activity, but were very slow and subject to bed collapse and deformation under minimal pressure or mobile phase variation. These two major deficiencies led to the use of silica as a rigid backbone for the chromatography [1–3] because good quality silica was available with appropriate pore and particle characteristics.

The excellence of the carbohydrate soft gels in terms of biocompatibility of the chemistry and functional groups was recognized; therefore, bonded phases were then, and continue to be, modeled on those supports. Silica can be easily derivatized, is economical, and has high quality that continues to improve as high-performance liquid chromatography (HPLC) matures as a standard laboratory technique. Silica-based sorbents for HPLC of proteins have now developed into a second generation of silica and bonded phase excellence for the most commonly used modes: size-exclusion (SEC), ion-exchange (IEC), hydrophobic interaction (HIC) and reversed-phase chromatography [4–8]. When these HPLC techniques are used, optimization can be readily achieved by proper selection of column support and mobile phase. This article will discuss relevant parameters of the first three modes, which are intended to deliver intact proteins. It will then offer guidelines for achieving the full potential of HPLC for resolution, selectivity and recovery of proteins.

2. SIZE-EXCLUSION CHROMATOGRAPHY

In protein chemistry, SEC is frequently used as a first step in a purification scheme to fractionate the proteins of interest from molecules of different sizes. SEC is also an easy technique for giving information pertaining to the size and shape of a molecule or for changing the buffer environment of a protein, often by desalting.

2.1. Bonding chemistry

To be suitable for SEC of proteins, silica supports must be well covered with a hydrophilic organic layer so that there is no contact or interaction with the surface silanols which can denature proteins. This has generally been achieved with glycerylpropylsilane [1] or a neutral polymer [2]. The resultant bonded phases are neutral but have mildly hydrophobic characteristics under certain mobile phase conditions, *e.g.*, high salt concentrations [9]. Silica-based SEC supports generally have a slight residual negative charge [9]. Manufacturers of high-performance (HP) SEC supports have minimized both ionic and hydrophobic properties under standard conditions of 0.1 *M* salt at neutral pH. Representative HPSEC supports are listed in Table 1.

2.2. Pore diameter

For a homogeneous series of polymers, log molecular weight (MW) is linearly related to the distribution coefficient within the internal volume of the column [4].

TABLE 1

SELECTED SILICA-BASED HPSEC SUPPORTS

Product	Manufacturer	Ref.
SynChropak GPC 60, 100, 300	SynChrom	10
TSK SW 2000, 3000, 4000	Toya Soda	11
Zorbax GF 250, 450	DuPont	12

There is deviance from linearity as the distribution coefficient (K_d) approaches either 0 or 1, as seen in Fig. 1, a protein calibration curve for an SEC support with a 100 Å pore diameter. Deviation of individual proteins from linearity may reflect a difference in shape (all proteins are not perfectly spherical) or interaction with the matrix. Although silica-based SEC supports are available with pores from 50–4000 Å, the best support is that on which the proteins of interest elute on the linear portion of the curve. Many globular proteins are smaller than 200 000 dalton and are best run on 100 or 300 Å supports. Denatured proteins, on the other hand, have increased hydrodynamic volumes and are usually run on supports with larger pores.

The homogeneity of pore diameter is reflected in the slope of the calibration curve. Excellent homogeneity will result in a low slope with linearity over a wide range of distribution coefficients. The lower the slope, the higher the potential for resolving proteins which do not differ greatly in molecular weight [4].

The pore volume is also characteristic of the quality of an HPSEC support [4]. All of the separation occurs within the included volume; therefore, it is important to have adequate pore volumes. The practical physical limitation to pore volume is fragility to pressures used in operation or column packing. It is desirable to have sufficient theoretical plates and pore volume that a column yields a minimum peak capacity of at least 7–10 peaks.

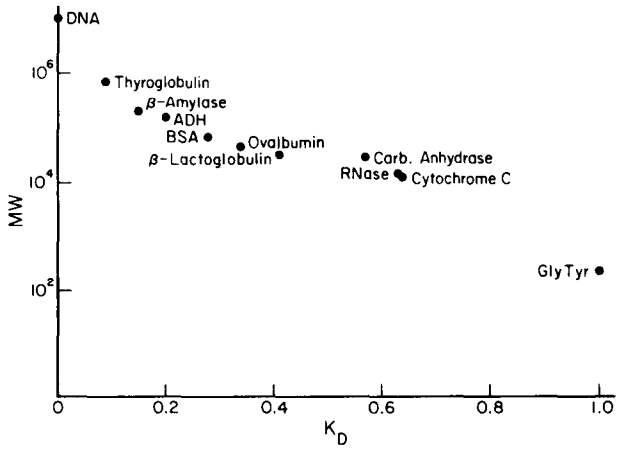

Fig. 1. Protein molecular weight calibration curve. Column: SynChropak GPC 100, 250 × 4.6 mm I.D. Mobile phase: 0.1 *M* potassium phosphate, pH 7. Flow-rate: 0.5 ml/min.

These requirements of the pore diameter, pore distribution and pore volume are only met by a limited number of commercial silicas which are used to produce HPSEC supports (Table 1). A major advantage of silica-based SEC supports is that pore characteristics do not change with mobile phase composition or pressure. As seen in Fig. 2, these HPSEC supports are capable of excellent separations of proteins by size.

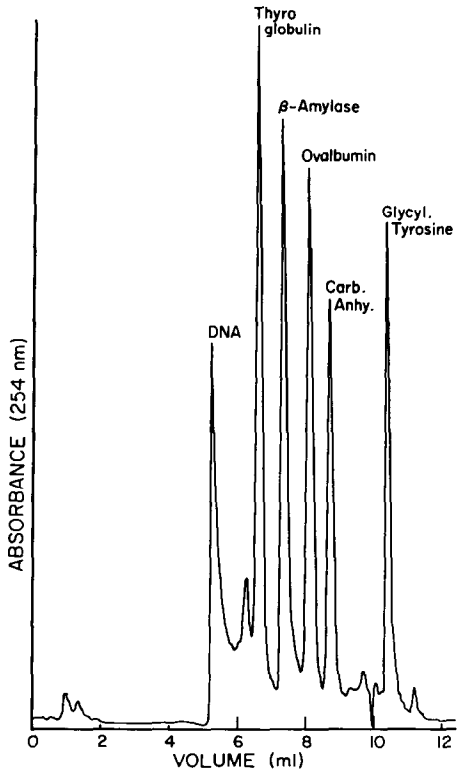

Fig. 2. HPSEC of proteins. Column: SynChropak GPC 300, 300 × 7.8 mm I.D. Mobile phase: 0.1 *M* potassium phosphate, pH 7. Flow-rate: 1 ml/min.

2.3. Particle size

To maximize resolution, HPSEC supports generally have particle diameters of 5–10 μm. A well packed column of microparticulate spherical HPSEC particles results in a minimal void volume and high efficiency, yielding maximal peak capacity. High plate counts for small molecules often can be achieved on silica-based SEC supports. It is important to recognize, however, that these high plate counts should not be presumed for proteins because their diffusion coefficients are so much lower. Additionally, mass transfer of proteins can be restricted if their dimensions approach those of the pores. Protein plate counts vary among different supports; this variance is probably related to the geometry of the pores and the relative ease of intraparticle diffusion.

The low diffusion coefficients of proteins necessitate the use of low flow-rates to obtain optimum resolution, even on well packed microparticulate HPSEC columns. Although microparticulate silica supports give high plate counts for small molecules over a wide range of linear velocities, linear velocities should not exceed 0.5 mm/s for protein or polymer analyses by SEC.

2.4. Mobile phase

Analysis times in SEC are short because the separation is performed isocratically. Due to the short contact time with the column, proteins usually remain stable if appropriate mobile phases and columns are used. Although pH should be between 2 and 8 due to the solubility of silica, this is generally not a limitation because it is also the normal range for protein stability. For silica-based SEC supports, optimum mobile phases usually contain 0.05–0.2 *M* salt. This level of salt is sufficient to compensate for the slight ionic nature of the support but it is not high enough to produce major hydrophobic interactions. For certain proteins, however, the mobile phase may require modification to eliminate any ionic or hydrophobic interactions. As is generally true, the mobile phase should primarily be chosen for its effectiveness in solubilizing and stabilizing the solutes. Certain proteins, such as membrane proteins, always need additives for solubilization [5]. Some additives which have been used successfully for HPSEC of proteins are glycerol [13], organic solvents [14,15] and detergents [5,16,17]. Because certain additives, such as detergents, may denature the protein, change its shape or increase its hydrodynamic volume, consideration of this fact is important in column choice and data interpretation [4]. Mobile phase additives do not affect the physical properties of silica-based SEC supports unless they chemically react with the bonded phase. This stability is an advantage over less rigid SEC supports which may deform when the mobile phase is changed.

2.5. Loading capacity

SEC has the lowest loading capacity of the modes of chromatography used for protein analysis due to its nonbinding characteristic and the fact that the separation occurs within the internal volume of the support. Approximately 2–4 mg of protein can be run without significant bandspreading on an HPSEC column which is 300 × 7.8 mm I.D. The volume of injection is similarly limited to about 2% of column volume.

2.6. Evaluation of HPSEC supports

To properly evaluate an HPSEC support for protein analysis, standard proteins, such as those seen in Fig. 1, should be run in a suitable buffer. The linearity of the calibration curve will show its suitability for size discrimination under those operating conditions. The slope of the linear portion of the calibration curve and the peak widths will determine the potential resolution of the column. It is best to follow the manufacturer's guidelines when choosing initial mobile phases and operating conditions for evaluation because differences in the physical or chemical properties of SEC supports may result in differences in optimal mobile phase conditions.

3. ION-EXCHANGE CHROMATOGRAPHY

Intrinsic charge properties are a major basis for protein purification. Proteins are amphoteric molecules which have specific p*I* characteristics and can be separated well by IEC. IEC is frequently used as a technique early in a purification scheme because it has high loading capacity and can remove many contaminants which vary in charge. This is especially important in biological fluids where the protein of interest may be in very small concentrations compared to substances such as albumin. The commercial IEC supports used for HPLC of proteins have been designed to offer excellent resolution (as seen for hemoglobins in Fig. 3), along with quantitative enzymatic or biological recoveries. They can be run with mobile phase conditions similar to those used with classical supports and reconditioned rapidly, thus yielding fast analysis times.

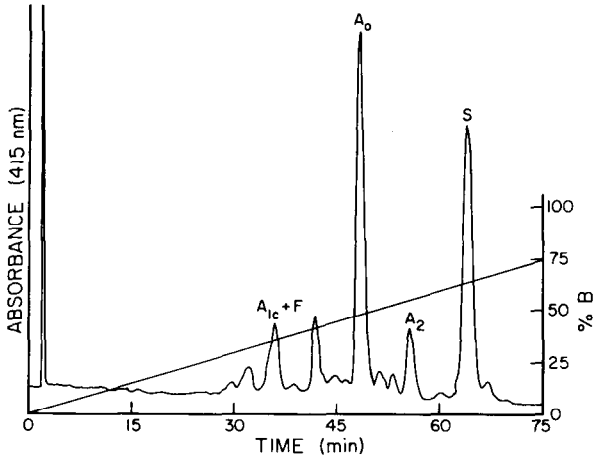

Fig. 3. HPIEC of human hemoglobins. Column: SynChropak CM 300, 250 × 4.6 mm I.D. Buffer: 0.03 *M* BisTris, 0.0015 *M* potassium cyanide, pH 6.4. 100 min gradient to 0.15 *M* sodium acetate. Flow-rate: 1 ml/min.

3.1. Bonding chemistry

Silica-based IEC supports for protein analysis are synthesized with a polymeric pellicular layer containing appropriately charged groups [2,6,18,19]. The layer must be very thin to give good mass transfer, totally cover the silica surface and not impede access to the pores. Simple silane derivatization is not suitable because such supports are unstable in aqueous buffers and do not totally cover the silica surface, resulting in exposed sites of charged silanol groups. Ion-exchange properties are determined by the specific ionic moieties incorporated into the polymer. Anion-exchange supports are generally made with diethylaminoethyl (DEAE), polyethyleneimine (PEI) or quaternary ammonium (QAE) functional groups. The DEAE and PEI are denoted "weak" because their charge varies with pH whereas the QAE is "strong" because it does not. Cation-exchange supports for HPLC generally incorporate carboxyl (CM) or

sulphonyl (SP) groups for "weak" or "strong", respectively. Neutral carbohydrate-like molecules are used for crosslinking, producing very stable bonded phases and creating a protective layer on the silica surface. Examples of commercial HPIEC supports can be seen in Table 2.

Most IEC supports exhibit a mild hydrophobicity due to the crosslinker used to form the polymeric layer. Generally, this hydrophobicity is only noticeable when high salt concentrations of chaotropic salts are intentionally used to produce multimodal interactions [20].

TABLE 2

SELECTED SILICA-BASED HPIEC SUPPORTS

Product	Functional group	Pore diameter (Å)	Manufacturer
Weak anion-exchange			
Bakerbond WP-PEI-WAX	PEI	300	J. T. Baker
SynChropak AX	PEI	100, 300, 1000	SynChrom
TSK DEAE-3-SW	DEAE	250	Toso Haas
Zorbax WAX	EA	300	DuPont
Strong anion-exchange			
SynChropak Q	Q	100, 300, 1000	SynChrom
Zorbax SAX	Q	300	DuPont
Weak cation-exchange			
Bakerbond CEX	CM	300	J. T. Baker
Poly CAT-A	Polyaspartic acid	300	Poly LC
SynChropak CM	CM	100, 300, 1000	SynChrom
TSK CM-3-SW	CM	250	Toso Haas
Zorbax WCX	CM	300	DuPont
Strong cation-exchange			
SynChropak S	S	300, 1000	SynChrom

3.2. Particle characteristics

Microparticulate silica-based supports (5–10 μm) give very good separations and purification for many proteins, even at relatively high loads (20 mg on a 250 × 4.6 mm I.D. column). Peaks are generally narrow under gradient conditions, as seen in Fig. 3. In situations where there is high risk of fouling, such as under high load conditions at an early stage of purification, particles larger than 10 μm can be effectively used for economical reasons. Resolution on such supports can be similar to that on microparticulate supports if appropriate changes in gradient slope and mobile phase velocity are made [21].

Pore diameters in IEC must be large enough to accomodate the solute protein so that it has access to the charged functional groups on the support. One major advantage of IEC as a technique is its high loading capacity. To optimize the loading capacity for proteins, the effective surface area must be maximized, usually by using the smallest pore diameter which will totally admit the protein. For proteins smaller

than 200 000 dalton, 300 Å pores give both excellent resolution and loading. At a flow-rate of 1 ml/min, the dynamic loading capacity for ovalbumin (MW 45 000) on 300 Å SynChropak AX 300 is 6.6 mg/ml and the absolute capacity is 80 mg/ml. For very large proteins, pores of 1000 or 4000 Å may be preferred for optimal loading.

3.3. Mobile phase

As in SEC, a primary consideration for mobile phase selection is solubility and stability of the protein solute. Because of proteins' amphoteric nature and multi-site binding, gradients must generally be used to resolve protein mixtures optimally and reproducibly. The initial buffer has a low ionic strength at a pH at which the protein is appropriately charged and bound to the support. A gradient of increasing ionic strength is usually used to release proteins, although a pH gradient can also be effective. With most silica-based HPIEC supports, a gradient to 1 *M* salt will remove all protein or ionic substances without adversely affecting the column. A versatile feature of IEC of proteins is that selectivity can be altered by changing the salt used for gradient formation [22,23].

To a lesser extent, pH can be adjusted to change the charge of the protein and thus change relative retention [24]. As a general guideline, the pH of the mobile phase should be at least 0.5 pH units above the p*I* of the solute for anion-exchange and below for cation-exchange. Exceptions to this rule exist primarily because the p*I* reflects all of the amino acids in the protein but only the surface amino acids are involved in the ion-exchange process [25]. For weak ion-exchange supports, pH will also determine the extent of ionization of the functional groups [24]. Due to their mechanical and chemical stability, silica-based HPIEC supports are easily washed and reconditioned in a variety of buffers which makes screening for an optimum mobile phase relatively easy.

It may be necessary to use additives to the mobile phase to enhance solubility or biological recovery. For example, EDTA can protect enzymes from metal impurities or β-mercaptoethanol can protect proteins from oxidation. Surfactants can be used to enhance solubility [26], but they should be neutral like Triton X-100 to be easily removed [5]. It should be recognized that detergents may change the ion-exchange characteristics of proteins by exposing additional amino acids to the support. Organic solvents may also be used for elution [27], but care must be taken if combined with salts so that precipitation can be avoided. Again, the stability of silica-based supports allows many choices in mobile phase selection and column configuration which makes HPIEC a very versatile technique.

4. HYDROPHOBIC INTERACTION CHROMATOGRAPHY

HIC is used to differentiate proteins by their surface hydrophobicity. The mild conditions for analysis allow preservation of biological activity and do not generally disrupt the tertiary structure. This relatively new HPLC method is becoming popular as an adjunct method for analyzing synthetic proteins and peptides [7]. It can achieve excellent resolution of proteins, as seen for calcitonin analogues in Fig. 4 [28].

4.1. Bonding chemistry

The bonded phases of reversed-phase chromatography and HIC supports are totally different and thus produce radically different selectivity. Silica-based reversed-

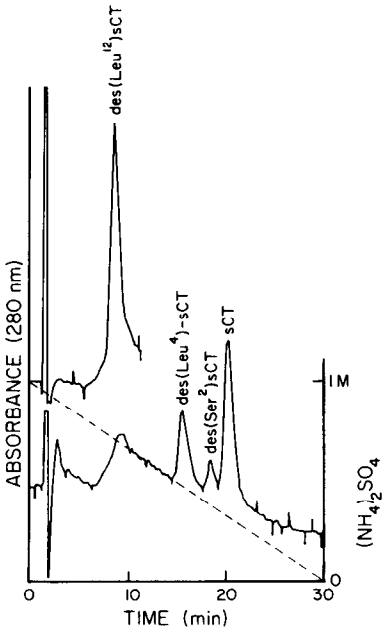

Fig. 4. HPHIC of calcitonin analogues. Column: SynChropak Propyl, 100 × 4.6 mm I.D. Buffer: 0.02 *M* phosphate, pH 7. 30 min gradient from 1 *M* to 0 *M* ammonium sulphate. Flow-rate: 1 ml/min [28].

phase supports have alkylsilane ligands and generally some residual silanols. The surface of HIC supports is totally covered with a thin layer of a hydrophilic polymer. Into this polymer are incorporated alkyl or aryl groups with a low ligand density compared to reversed-phase. The length of the hydrophobic ligands on the HIC support greatly affects retention and selectivity [29] whereas it does not in reversed-phase chromatography [7]. Hydrophilic ligands, such as hydroxypropyl, only retain extremely hydrophobic molecules, whereas longer chains, such as pentyl, retain even hydrophilic proteins strongly. The bonded phase of silica-based HIC supports is very stable, allowing versatility in mobile phase selection and cleaning procedures. Table 3 lists some commercially available silica-based HPHIC supports.

TABLE 3

SILICA-BASED HPHIC SUPPORTS

Support	Pore size (Å)	Phase	Manufacturer
BakerBond HI-propyl	300	Propyl	J. T. Baker
Spherogel CAA-HIC	300	Methyl polyether	Beckman
Polyethyl A, Polypropyl A	330	Polyalkylaspartamide	Poly LC
LC-HINT		"Polar" bonded phase	Supelco
SynChropak Methyl, SynChropak Propyl, SynChropak Pentyl, SynChropak Benzyl, SynChropak Hydroxypropyl	300, 1000	Alkyl polyamide	SynChrom

4.2. Particle characteristics

In HIC, as in IEC, it is necessary to use a pore large enough to give access to the protein so that it can interact with the ligands. Most silica-based HIC supports have 300 Å pores, although larger ones are available. Because the loading capacity is related to the effective surface area, it will be maximized when the pore diameter is slightly larger than the protein. HPHIC loading capacities are similar to those for HPIEC. For ovalbumin, the dynamic loading capacity at 1 ml/min is 5 mg/ml on 300 Å SynChropak Propyl.

4.3. Mobile phase

HPHIC is performed with a reverse salt gradient, usually composed of ammonium sulphate in a phosphate buffer. Several studies have examined the effects of different salts or pH on resolution and selectivity in HIC [30–32]. pH has only a minor effect, probably related more to solubility of the protein than to the chromatography. Chaotropic salts, such as sodium and ammonium sulphate, are most effective at causing hydrophobic interactions.

Mobile phase additives to enhance solubility or stability may be used effectively in HIC on silica-based supports. Surfactants have also been used successfully [33]. The mechanical rigidity and stability of silica-based supports is particularly important in HIC because the high salt concentrations can produce higher pressures or cause bed deformation in softer gels.

5. CONCLUSIONS

Silica-based HPLC media are excellent for protein purification in terms of resolution, recovery and speed of analysis. Silica supports can be easily derivatized with a variety of bonded phases. Silica particles are available in appropriate pore diameters and pore volumes for optimum analysis of proteins. The rigid particles are stable to many mobile phases, including 0.1% trifluoroacetic acid, high salt concentrations and organic solvents which can be used for elution or cleaning purposes.

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