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A comprehensive study to protein retention in hydrophobic interaction chromatography

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Highlights

- Selectivity is governed by the salt concentration and molal surface tension increment
- The hydrophobicity index can be used to predict the elution order of intact proteins
- HIC can be used to profile 3D conformational changes.

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Abstract

The effect of different kosmotropic/chaotropic salt systems on retention characteristics of intact proteins has been examined in hydrophobic interaction chromatography (HIC). The performance was assessed using different column chemistries, *i.e.,* polyalkylamide, alkylamine incorporating hydrophobic moieties, and a butyl chemistry. Selectivity in HIC is mainly governed by the salt concentration and by the molal surface tension increment of the salt. Typically, a linear relationship between the natural logarithm of the retention factor and the salt concentration is obtained. Using a 250 mm long column packed with 5 μm polyalkylamide functionalized silica particles and applying a 30 min linear salt gradient, a peak capacity of 78 was achieved, allowing the baseline separation of seven intact proteins. The hydrophobicity index appeared to be a good indicator to predict the elution order of intact proteins in HIC mode. Furthermore, the effect of adding additives in the mobile phase, such as calcium chloride (stabilizing the 3D conformation of α-lactalbumin) and isopropanol, on retention properties has been assessed. Results indicate that HIC retention is also governed by conformational in the proteins which affect the number of accessible hydrophobic moieties.

Keywords*:* Salting-out chromatography; Hofmeister; Hydrophobicity; Monoclonal antibodies; Conformational change

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1. Introduction

The need for a comprehensive characterization of protein-derived macromolecules used in the biopharmaceutical and food industries is increasing rapidly [1,2]. More efficient analysis of new modified products may improve and accelerate innovation and may be translated into safer products and improved production processes. Liquid chromatography (LC) presents many exciting possibilities for the characterization of complex samples. Various forms of LC exist, allowing separation of sample constituents according to their polarity (normal-phase LC or reversed-phase LC), hydrodynamic volume (size-exclusion chromatography), charge in solution (ion-exchange chromatography), *etc*. Whereas denaturing LC conditions are typically applied, native protein separation technology maintains the 3D protein conformation [3]. Examples of native LC modes include aqueous size-exclusion chromatography [4], aqueous ion-exchange chromatography [5], and hydrophobic interaction chromatography (HIC) [6].

HIC capitalizes on the interaction between hydrophobic patches of proteins and weakly hydrophobic ligands attached to the stationary phase [7,8]. The separation is typically performed using aqueous (non-denaturing) buffer systems at pH = 7 and applying a linear salt gradient starting at relatively high salt concentration [9]. The origin of HIC technology can be traced back to 1948 when Shepard and Tiselius discussed the adsorption of proteins on silica gel in the presence of salt, called "*salting-out chromatography*" [10]. Other landmark contributions include the work of Shaltiel and Er-El, discussing protein retention by lipophilic interactions between accessible hydrophobic pockets of proteins with carbon side chains on the stationary phase [11], and the seminal work of Horvath *et al.* who developed a theoretical framework describing the effects of salt on hydrophobic and electrostatic interactions [12]. Since then most efforts have been directed to elucidating the retention mechanism of HIC and demonstrating the application possibilities of the technology.

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Extensive studies have been conducted to investigate mobile-phase [13-16] and stationary-phase contributions to protein retention [17,18]. It has been reported that the influence of nature of salt on retention is governed by the Hofmeister series [19]. Kosmotropic salts have higher polarity than chaotropic salts and interact with water strongly. This leads to the formation of strong hydration layer around the kosmotropic salt, hence leaving the hydrophobic patches of the stationary phase unexposed, promoting HIC interaction. Chaotropic salts disrupt hydrogen bonding and reduce the hydrophobic effect (destabilizing the native structure of the proteins) and therefore weaken the hydrophobic retention effects. However, recent studies have demonstrated that protein retention in HIC appears to be affected by an interplay of different contributions, such as pH [13,14], salt concentration and type [15,16], ligand type and ligand density [17,18], unfolding of proteins upon adsorption [20], kinetics of protein spreading [21], *etc*. Protein retention has also been linked to protein properties, including the hydrophobicity index. Different excellent reviews have appeared in the literature describing different approaches to determine hydrophobicity indexes [22-24] and retention-time models as function of different input parameters [25-27]. Fausnaugh and Regnier demonstrated the effect of amino-acid (AA) substitution on protein retention using lysozyme isolated from different bird species [28]. It was concluded that AA substitution on the protein surface affected the strength of the hydrophobic interaction rather than changing the contact area. Retention was furthermore influenced by the ionization state of histidine residues. More recently a number of key references have appeared in literature describing the application possibilities of HIC to analyze antibody variants [29,30]. Valliere-Douglass *et al.* described the application of HIC for assessing the heterogeneity, stability, and potency of monoclonal antibodies and Fab and Fc sub-domains [29]. An overview of application possibilities to profile therapeutic proteins with HIC was provided by Haverick *et al.* [30].

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Recently, the group of Guillarme and Fekete described practical aspects of mobile-phase optimization for method-development purposes [31,32]

Although HIC shows great potential for native biomolecule separations, the effects of operating conditions such as mobile phase composition (type and concentration of salts and the possibility to add organic modifiers), and stationary-phase chemistry on retention are still not fully understood. In this study, the performance of four commercially-available HIC columns was assessed for protein HIC separations and the effects of eluent type and concentration on protein retention was investigated using different kosmotropic/chaotropic salt systems, including ammonium sulfate, sodium sulfate, potassium sulfate, sodium chloride, and sodium nitrate dissolved in phosphate buffer pH = 7.0. Using optimized column and mobile-phase systems the possibilities to separate intact proteins were explored. Finally, effects of mobile-phase additives (calcium chloride and isopropanol) on retention has been studied.

2. Materials and methods

2.1. Chemicals and materials

Sodium dihydrogen phosphate (≥99.0%), disodium hydrogen phosphate (≥99.0%), sodium hydroxide (HPLC grade, 50.0%), sodium chloride (≥99.0%), ammonium sulfate (≥99.0%), sodium sulfate (≥99.0%), potassium sulfate (≥99.0%), sodium nitrate (≥99.0%), and calcium chloride hexahydrate (98%), cytochrome *c* from bovine heart, myoglobin from equine heart, ribonuclease A from bovine pancreas, apo-transferrin from bovine pancreas, lysozyme from chicken egg white, trypsinogen from bovine pancreas, α-chymotrypsinogen A from bovine pancreas, α-chymotrypsin from bovine pancreas, calcium-depleted α-lactalbumin from bovine milk, and bovine serum albumin (BSA) from bovine pancreas were

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purchased from Sigma-Aldrich (Diegem, Belgium). Isopropanol (LC-MS grade) was purchased from Biosolve (Dieuze, France). Deionized HPLC-grade water was produced in-house using a Milli-Q water purification system (Millipore, Molsheim, France).

100 mm × 4.6 mm i.d. HIC columns (ProPac HIC-10, MAbPac HIC-20, MAbPac HIC-10, and MAbPac HIC butyl) and a 250 mm × 4.6 mm i.d. MAbPac HIC-20 column were provided by Thermo Fisher Scientific (Sunnyvale, USA). The stationary-phases properties are described in Table I.

2.2. Instrumentation

HPLC experiments were conducted using an UltiMate 3000 HPLC system (Thermo Fisher Scientific, Germering, Germany) equipped with a membrane degassed, ternary low-pressure-gradient pump, a thermostatted split-loop autosampler (set at 6°C), a forced-air column oven, and a diode-array detector equipped with a 3 µL UV flow cell (9 mm path length). 250 mm x 100 µm i.d. tubing was used to connect the autosampler to the column inlet, and to connect the column outlet to the UV flow cell. All isocratic and gradient separations were performed in duplicate applying a flow rate of 1 mL/min, 3 µL injection volume, a column oven temperature of 30ºC, and UV detection at λ = 230 nm with a data collection rate of 50 Hz and a response time of 0.2 s.

Differential scanning calorimetry (DSC) was performed on a TA Instruments (DE, USA) nano-DSC III instrument between 25 and 90°C with a scanning rate of $1°C{\cdot}min^{-1}$ at 3 atm. The capillary cell (V = 300 mL) was filled with the α-lactalbumin solution, final concentration 2 $mg{\cdot}mL^{-1}$ in a 50 mM PBS buffer at pH 7 containing 1.2 M of $(NH_4)_2SO_4$. Experiments were run in the absence of any added reagents, with 5% isopropanol or with 5% isopropanol and 10 mM $CaCl_2$. The reference cell was filled with the corresponding protein-

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free solution. The samples were degassed for 7 minutes prior to measurement. For each sample, at least four cycles of heating and cooling were performed with 10 minutes of thermal equilibration between the ramps. The thermograms were processed and analyzed using NanoAnalyze software from TA Instruments.

2.3. Mobile-phase and sample preparation

Mobile phase A was prepared by dissolving the desired salt concentration (2 M sodium sulfate, 0.7 M potassium sulfate, 2 M ammonium sulfate,5 M sodium chloride, or 2 M sodium nitrate) in a 0.05 M disodium hydrogen phosphate/sodium dihydrogen phosphate solution. The pH of the mobile-phases was adjusted to pH = 7.0 by the addition of 2 M sodium hydroxide solution. The ionic strength was adjusted using mobile phase B, which consists of 50 mM phosphate buffer pH = 7.0. Additionally, the effect of adding organic modifier to mobile phase A on solute retention was studied by adding 2.5% (v/v) isopropanol to 50 mM phosphate buffer containing 1.2 M ammonium sulfate and 10 mM calcium chloride. Prior to use mobile phases were filtered over 10 μm Polypropylene filter (Gelman Sciences, Ann Arbor, USA).

Samples for isocratic retention-time measurements were prepared by dissolving proteins in 50 mM phosphate buffer containing one of the salt types, *i.e.*, 1 M ammonium sulfate, 1 M sodium sulfate, 0.7 M potassium sulfate, or 2.5 M sodium chloride. The concentration of the proteins were 2 mg/mL for ribonuclease A, myoglobin, and BSA, and 4 mg/mL for α-chymotrypsin. For the gradient separation, a protein mixture containing cytochrome *c*, myoglobin, ribonuclease A, apo-transferrin, lysozyme, trypsinogen, and α-chymotrypsinogen A was prepared in 50 mM phosphate buffer pH = 7.0 containing 1.8 M ammonium sulfate. The concentration of proteins in solution was 4.8 mg/mL.

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3. Results and discussion

3.1. Effect of salt concentration and type on protein retention

The effect of salt concentration on retention characteristics was determined by injection of individual proteins and applying isocratic LC conditions at a fixed flow rate of 1.0 mL/min. Ammonium sulfate was used as salt, dissolved in phosphate buffer pH = 7.0. Fig. 1A shows the resulting chromatograms recorded for ribonuclease A. Gaussian peak profiles are observed and peak width increases proportionally to retention time. At higher salt concentration, the hydrophobic patches at the stationary-phase surface are readily accessible, leading to increased protein retention. The retention behavior of four model proteins (myoglobin, ribonuclease A, BSA, and α-chymotrypsin A) are displayed in Fig. 1B. At pH = 7 myoglobin, ribonuclease A and α-chymotrypsin A are positively charged, whereas BSA will behave as an anion. A similar visualization approach was selected, *i.e.*, the logarithm of the retention factor (ln k) *versus* the molal salt concentration, as proposed by Snyder and Dolan in the 1980's for retention-time modelling of reversed-phase LC separations using aqueous organic modifiers as the mobile phase [33]. The proteins display linear retention-time behavior, which can be described by:

$$ln(k) = ln(k_w) - S \cdot [M] \qquad (1)$$

where k_w is the extrapolated value of k for $[M_0]$, *i.e.*, in pure buffer and S is the solvent-strength parameter, which is a constant for a given protein. Table II summarizes the M_r, pI, and hydrophobicity-index values (ϕ_S) of all proteins used in this study. For the determination of ϕ_S, it is assumed that each amino acid situated at the surface of the protein has a hydrophobic contribution proportional to its solvent accessible area [34]. Therefore, files from the protein data bank (PDB) incorporating information such as the amino-acid

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sequence, stoichiometry, secondary-structure locations, crystal lattice, and symmetry group in the crystal of protein, have been used as input in the GETAREA software from Fraczkiewicz and Brown to calculate the solvent accessible area per amino-acid residue [35]. The ϕ_S for each protein was then calculated by incorporating the normalized Miyazawa–Jernigan amino-acid hydrophobicity scale [36]. Fig. 1B shows that the hydrophobicity index generally seems to be a good indicator to predict the elution order of intact proteins in HIC mode. Typically, higher salt concentrations are required to elute proteins that exhibit lower ϕ_S.

Protein retention was assessed using different kosmotropic and chaotropic salt systems, including, sodium, potassium, and ammonium sulfate, sodium nitrate, and sodium chloride in phosphate buffer pH = 7.0. Fig. 2A shows the effects of salt concentration, for the different salt systems, on the retention factor for ribonuclease A, applying the different salt systems. Sodium nitrate was excluded from the experiments because of the high UV background signal. Furthermore, only a limited number of data points was acquired using potassium sulfate, due to limitations in solubility. Using kosmotropic salt systems such as ammonium, potassium, or sodium sulfate hydrophobic, interactions are promoted, resulting in higher retention factors and a steeper curve, in contrast to a chaotropic salt such as sodium chloride. Hence, to generate enough retention for HIC protein separations using monovalent sodium chloride, relatively high salt concentrations are required. Although the Hofmeister series indicates that retention should increase when replacing sodium by potassium and potassium by ammonium cations [19], the opposite trend was observed.

Changing the salt concentration and type affects the surface tension of the mobile phase at the surface of the stationary-phase particles. Furthermore, most likely the 3D structure and hence surface properties will be affected. The sum of these effects will in turn affect protein retention. With increasing salt concentration the surface tension increases, which effectively decreases protein retention, as demonstrated in Fig. 2. Furthermore, the

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retention order of the different proteins when using different salt types is linked to the molal surface-tension increment (σ), which is in agreement with the results reported previously by Horvath *et al.* [12]. Sodium sulfate with $\sigma = 2.73$ mM·m^{-1}·cm^{-1} yielded the highest retention factors, and protein retention decreases with σ, *i.e.,* $\sigma = 2.58$ mM·m^{-1}·cm^{-1} for potassium sulfate, 2.16 mM·m^{-1}·cm^{-1} for ammonium sulfate, and $\sigma = 1.64$ mM·m^{-1}·cm^{-1} for sodium chloride yielding the lowest retention factors. This trend was confirmed for the different proteins (data not shown).

The performance of the different columns listed in Table I was scouted for HIC separations. Fig. 3 shows the retention-time behavior for ribonuclease A (Fig. 3A) and α-chymotrypsin (Fig. 3B). The ProPac HIC-10 and MAbPac HIC-20 columns displayed similar retention-behavior (similar slope), which can be expected since the resins have similar polyalkylamide surface chemistries. Due to the larger accessible surface area the ProPac HIC-10 column yields higher retention factors. The magnitude seems to be protein dependent, since the difference in retention factor for ribonuclease A is much larger than that observed for α-chymotrypsin. A possible explanation may be that α-chymotrypsin is partly excluded from the stagnant pores when using the 300Å resin. The MAbPac HIC-10 with alkylamide functionality incorporating hydrophobic moieties displays a different slope, indicating different selectivity compared to the ProPac HIC-10 and MAbPac HIC-20 columns. In case of ribonuclease A, slightly higher retention was observed, compared to the MAbPac HIC-20 (both columns exhibit the same 1000Å pores), especially when applying higher salt concentrations. However, for α-chymotrypsin the retention factors are significantly lower compared to the other columns tested in our study. A possible explanation for this retention behavior is that the surface chemistry affects the 3D conformation during the protein-stationary phase interaction, and this effect may be protein dependent. Jungbauer *et al.* also discussed the possibility that (partial) unfolding of proteins occurs upon adsorption

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on the stationary phase, and proposed an empirical retention-time model taking this effect into account [20]. The column packed with non-porous particles coated with butyl moieties (MAbPac HIC-butyl) displays essentially the same retention factors compared to the columns packed with alkylamide functionality incorporating hydrophobic moieties. On the MAbPac HIC-butyl the retention factors for ribonuclease A are slightly lower, but α-chymotrypsin displays slightly more retention than the MAbPac HIC-20 column. It is interesting to note that the relationship between ln k and salt concentration on the MAbPac HIC-butyl appears to be non-linear, *i.e.*, the R^2 using linear regression was determined to be 0.9195, whereas the R^2 using a quadratic model fit was determined to be 0.9576. Non-linear retention behavior may indicate (but is not necessarily caused by) the presence of a dual or multi-mode retention mechanism [37]. In this case, the non-porous methacrylate particles functionalized with butyl groups do not contain ionizable moieties. Hence, the non-linear behavior cannot be explained by secondary electrostatic interactions.

3.2. Profiling of intact proteins and conformation changes

The gradient separation of a mixture of 7 intact proteins performed on the MAbPac HIC-20 column is depicted in Fig. 4A. The protein properties, *i.e.*, MW, *pI*, and ϕ_S are provided in Table II. A linear gradient of ammonium sulfate (dissolved in 50 mM phosphate buffer pH = 7.0) was applied with a gradient time of 30 min. All proteins are baseline resolved and the peak capacity based on the gradient time and 4-sigma peak width of myoglobin (W = 0.387 min) was determined to be 78. Compared to, for example, myoglobin or ribonuclease A, apo-transferrin yields a relatively broad peak. Apo-transferrin is a very heterogeneous protein and the peak profile may possibly represent different protein isoforms due to glycosylation.

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The 3D conformation of proteins can be strongly affected by the composition of the mobile-phase. In addition, protein adsorption to the stationary-phase surface may induce conformational changes [20,21]. To assess possible conformational changes induced by the mobile-phase composition affecting retention, HIC experiments were conducted in isocratic mode using α-lactalbumin as test analyte. Fig. 5A shows the peak profiles that were obtained by injecting α-lactalbumin using conventional (isocratic) HIC conditions, *i.e.*, ammonium sulfate in phosphate buffer). Adding 2.5% isopropanol to the mobile-phase containing ammonium sulfate salt in phosphate buffer resulted in the elution of a very broad peak with a peak top eluting around 40 min, which can hardly be distinguished from the baseline noise, see Fig. 5B. DSC experiment (Fig. 6) demonstrate that the addition of a small amount of organic modifier leads to the denaturing of α-lactalbumin making more hydrophobic moieties accessible for interaction with the stationary phase. Thermograms clearly highlight the destabilizing effect of isopropanol, lowering the T_m by 13°C and the denaturation enthalpy compared to the sample in the absence of organic modifier.

α-lactalbumin has a single strong calcium binding site, which is formed by the carboxylic groups of thee Asp residues and two carbonyl groups in a loop between two helices [38]. As such the addition of calcium to the sample or mobile phase can stabilize the 3D structure of the protein. Adding 10 mM calcium chloride to the sample solution or mobile-phase while maintaining the same mobile-phase composition did not significantly affect the retention time (data not shown). Although, calcium chloride is a known chaotropic salt, however to affect retention relatively high salt concentration (>> 1 M) should be employed. However, when conducting a similar experiment and adding calcium chloride to the mobile phase containing 5% isopropanol ammonium sulfate in phosphate buffer, the retention time of α-lactalbumin elutes is significantly decreased, see Fig. 5C. The addition of 10 mM calcium chloride in the mobile phase (and sample) stabilizes the 3D protein

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conformation, as observed in the DSC thermogram; the salt leads to a 5°C T_m increase (Fig. 6). Only a limited number of hydrophobic moieties will be accessible for interaction with the stationary phase. Due to the addition of isopropanol in the mobile-phase the solvent strength is increased, which leads to a decrease in retention time.

4. Concluding remarks

The results show that the main characteristics affecting protein retention in HIC include concentration and type of the salt, chemistry of hydrophobic ligand attached to the stationary-phase surface, and the physical properties of the protein. The effects of salt and column chemistry on retention is a complex phenomenon that includes the surface tension of the salt solution, physicochemical properties of the salt, and the nature of protein. The effect of salt type on protein retention can be related to the concentration and molal surface tension increment of salt. Salts with higher molal surface tension increments increase retention when applying equal molal salt concentration. Sodium sulfate resulted in the highest retention factor. Moreover, differences in selectivity were observed when sodium sulfate was used in comparison to monovalent salt such as sodium chloride. The linear retention time behavior ($\ln k$ *versus* $[M]$) allows the use of the LSS model proposed by Snyder and Dolan to realize a generic method-develop strategy for HIC.

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Figure Captions

Figure 1. Effect of salt concentration on protein retention. (A) Isocratic elution profiles of ribonuclease A obtained varying the ammonium sulfate concentration between 0.7 and 1.5 M. (B) Relationship between retention factor and salt concentration measured for myoglobin (circle), ribonuclease A (triangle), BSA (diamond), and α-chymotrypsin (square). Mobile phase: ammonium sulfate in 50 mM phosphate buffer pH = 7.0. Flow rate = 1 mL/min. Column temperature = 30°C.

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Figure 2. Effect of salt concentration and type on the retention of ribonuclease A. Mobile phases: sodium sulfate (triangle), potassium surface (square), ammonium sulfate (circle), and sodium chloride (diamond) in 50 mM phosphate buffer pH = 7.0.

Figure 3. Retention of ribonuclease A (A) and α-chymotrypsin (B) as function of salt concentration measured on columns with different chemistries and pore sizes. Columns: ProPac HIC-10 (diamonds), MAbPac HIC-20 (circles), MAbPac HIC-10 (squares), and MAbPac HIC-butyl (triangles). Mobile phase: ammonium sulfate in 50 mM phosphate buffer pH = 7.0. Flow rate = 1 mL/min. Column temperature = 30°C.

Figure 4. Gradient separation of intact proteins on a 250 mm long MAbPac HIC-20 column and applying a 30 min linear gradient of ammonium sulfate in 0.5 M phosphate buffer pH = 7.0. Peak identification: (1) cytochrome *c*, (2) myoglobin, (3) ribonuclease A, (4) apo-transferrin, (5) lysozyme, (6) trypsinogen, (7) α-chymotrypsinogen A.

Figure 5. Peak profiles of α-lactalbumin obtained in isocratic mode showing the effect of the addition of additives (calcium chloride and isopropanol) in the mobile phase on the retention. Separations were performed on a 250 mm long MAbPac HIC-20 column applying (A) 1.2 M ammonium sulfate in 50 mM phosphate buffer, (B) 1.2 M ammonium sulfate in 50 mM phosphate buffer and 5% IPA, and (C) 1.2 M ammonium sulfate in 50 mM phosphate buffer and 2.5% IPA and 10 mM calcium chloride as the mobile phase. (Fig. 5B Protein amount was increased for peak detection).

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Figure 6. Differential scanning calorimetry thermograms (in heating mode) of (a) a 2 mg·mL^{-1} α-lactalbumin solution in 50 mM PBS buffer at pH 7 and 1.2 M of $(NH_4)_2SO_4$ (solid line), (b) same as A with 5% isopropanol (dashed line), (c) same as B with 10 mM $CaCl_2$.

Tables

Table I. Summary of stationary-phase properties.

Column	ProPac HIC-10	MAbPac HIC-20	MAbPac HIC-10	MAbPac HIC-butyl
Material	silica	silica	silica	polymethacrylate
Particle size (µm)	5	5	5	5
Pore size (Å)	300	1000	1000	non porous
Surface area (m^2/g)	100	20	20	1.36
Surface chemistry	polyalkylamide	polyalkylamide	alkylamide	butyl

Table II. Physical properties of intact proteins.

Protein	M_r	*pI*	ϕ_S
cytochrome *c*	12,200	10.60	0.210
myoglobin	16,951	8.13	0.214*
ribonuclease A	13,574	9.77	0.230
apo-transferrin	77,000	5.50	0.239**
bovine serum albumin	68,000	4.4 – 4.8	0.240
lysozyme	14,000	11.0	0.278
trypsinogen	23,700	9.30	0.289
α-chymotrypsin	25,207	9.69	0.299
α-chymotrypsinogen A	25,600	8.97	0.306

*value based on the three dimensional structure of metmyoglobin (oxidized form of myoglobin)

**value based on apo-human serum transferrin (glycosylated)