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Dextran-grafted silica gel for high-performance size-exclusion chromatography of proteins

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Abstract

A hydrophilic stationary phase for aqueous high-performance size-exclusion chromatography (HPSEC), *viz.*, dextran-grafted silica gel, was synthesized and tested. Using dextran or dextran derivatives covalently grafted on to epoxysilica gel, it was possible to achieve good hydrophilic shielding of the silica gel surface, preserving the unique physical properties of silica matrix. The retention of proteins on the stationary phase was studied to optimize the modification procedure. The undesired silica matrix effect was minimized by combination of three phenomena: chemical removal of silanols by silanization, steric shielding of residual silanols with grafted macromolecules and electrostatic compensation of SiO^- groups by a positively charged anchoring group. Optimized dextran-grafted silica is a suitable packing material for the HPSEC of proteins, operating with a wide range of ionic strength of the eluent.

1. Introduction

Numerous silica-based packing materials for aqueous high-performance size-exclusion chromatography (HPSEC) with high pressure stability and excellent flow properties have been prepared [1]. The main problem is that they often show undesirable interaction of residual surface silanols with many solutes [2].

To be suitable for the SEC of biopolymers, such as proteins, silica supports must therefore be well covered with a hydrophilic organic layer. This has been done mainly by the reaction of silanols on the silica surface with γ-glycidyloxypropyltrimethoxysilane to produce diol-bonded phases [3]. Better shielding of residual silanols has been achieved by the immobilization of hydrophilic organic macromolecules, such as polyvinylpyrrolidone [4], poly(ethylene oxide) [5], polysaccharides [6–9] and other polymers [10,11], on the silica gel. Additionally, the unfavourable interactions of proteins with residual silanols can be prevented by the presence of positively charged groups linked to the immobilized polymer, *e.g.*, diethylamino groups on dextran [8].

None of the individual approaches, chemical removal of silanols, steric shielding of residual silanols or electrostatic compensation of SiO^- groups, fully solves the fundamental problem of the undesired solute retention on silica-based materials in aqueous SEC. Hence the combination of these approaches should be considered.

In this paper, the preparation and characteri-

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zation of a hydrophilic stationary phase for the HPSEC of proteins with minimized protein–sorbent interactions are described. The material was prepared by covalent grafting of dextran molecules (native or derivatized with ethylenediamine) on to the silica surface via epoxy groups previously bonded to the silica surface.

The main difference between the dextran-grafted silica gel and silica gel coated with a layer of cross-linked dextran [7–9] is that dextran grafts may protrude further over the walls of pores than does the more or less intact layer of macromolecules. Protruding dextran chains may fit with the native conformation of separated proteins preserving their biological activity.

As it is known that electrostatic interactions decrease and hydrophobic interactions increase with increasing ionic strength [12,13], the dependence of the retention of selected proteins on the salt content in the eluent was studied to characterize the nature and the intensity of undesirable solute–sorbent interactions. Such information was used for optimization of the grafting procedure. The resulting dextran-grafted silica gel with optimized properties was used for the HPSEC of model proteins.

2. Experimental

2.1. Materials

Spherical silica gel SG-10-78 (mean particle diameter 8 μm; specific surface area 78 $m^2\ g^{-1}$, specific pore volume 1.3 ml g^{-1}) was prepared by modification of the laboratory-made base silica material designated SG-10 [14] to obtain larger pores. γ-Glycidyloxypropyltrimethoxysilane was supplied by Fluka (Buchs, Switzerland). Dextran with a relative molecular mass of $4 \cdot 10^4$ (Rheodextran, D-40) was obtained from Biotika (Slovenská Lupča, Slovak Republic).

Ethylenediamine (99%) was obtained from Janssen Chimica (Bruges, Belgium), sodium periodate from Carlo Erba (Milan, Italy), sodium borohydride from Metallgesellschaft (Frankfurt a.M., Germany), 2,4,6-trinitrobenzoic acid, (analytical-reagent grade) from Serva (Heidelberg, Germany) and 6-aminocaproic acid (99%) from Aldrich (Milwaukee, WI, USA).

Aminoethyl derivatives of dextran (AE-dextran, AE-D) were prepared by reductive alkylation [15,16] of dextran dialdehyde with ethylenediamine and sodium borohydride [15]. The degree of substitution of AE-D was controlled by the molar ratio of sodium periodate to dextran, whereby the generated aldehyde groups were subsequently reductively alkylated in the presence of a large excess of diamine. Primary amino groups of AE-D derivatives were determined spectrophotometrically at 360 and/or 420 nm by a procedure based on the reaction of free amino groups with 2,4,6-trinitrobenzenesulphonate (TNBS) [17,18]. Molar absorptivities ε_{360} = 11 000 l mol^{-1} cm^{-1} and ε_{420} = 18 320 l mol^{-1} cm^{-1} were determined by reaction of 6-aminocaproic acid with TNBS.

The protein standards (Table 1) were obtained from Pharmacia–LKB (Uppsala, Sweden), Boehringer (Mannheim, Germany) and Sigma (St. Louis, MO, USA). D,L-Alanine and deuterium oxide were purchased from Lachema (Brno, Czech Republic). Dextran standards for SEC calibration with relative molecular masses from $1 \cdot 10^3$ to $6.7 \cdot 10^5$ were obtained from Pharmacosmos (Viby, Denmark) and dextran with relative molecular mass $2 \cdot 10^6$ from Pharmacia–LKB.

2.2. LC experiments

The chromatograph used for the LC characterization of sorbents consisted of a Waters Model 510 HPLC pump (Waters–Millipore, Milford, MA, USA), a Rheodyne (Cotati, CA, USA) Model 7120 injector, an RIDK 101 differential refractometric detector, a Model 2563 UV–Vis detector and a TZ 4620 line recorder (Laboratory Instruments, Prague, Czech Republic). A Baseline 810 chromatography workstation (Dynamic Solutions, Division of Millipore, Ventura, USA) based on an NEC SX plus computer (purchased from Waters) was used for data acquisition and processing.

HPSEC separations were performed on a

Table 1
Protein calibration standards

Standard	Abbreviation	Supplier[a]	$M_r \times 10^{-3}$ [b]	Source[b]
Cytochrome *c*	CYT	B	12.5	Horse heart
Ribonuclease	RN	P	13.7	Bovine pancreas
Chymotrypsinogen A	CHT	P, B, S	25	Bovine pancreas
Ovalbumin	OVA	P, B	43, 45	Hen egg
Albumin	BSA	P, B	67, 68	Bovine serum
Aldolase	ALDO	P, B	158	Rabbit muscle
Catalase	CAT	P, B	232, 240	Bovine liver
Ferritin	FR	P, B	440, 450	House spleen
Thyroglobulin	TG	P	669	Bovine thyroid

[a] P = Pharmacia; B = Boehringer; S = Sigma.
[b] As given by the supplier.

liquid chromatograph consisting of a Model 2150 HPLC pump, a Model 2140 rapid spectral detector (both from LKB, Bromma, Sweden), a Rheodyne Model 7010 injector and an Acer computer with HPLC Analysis Program Wavescan EG (purchased from LKB).

Model mixtures were prepared from 1.0 mg ml^{-1} stock standard solutions of individual standards. The injection volumes were 20 or 10 μl. Low-molecular-mass alanine or deuterium oxide was used as a marker for the determination of the total volume of liquid within the column. The void volume was determined by injection of the highest molecular mass dextran standard. Each standard was injected individually into the particular column to calibrate it or to identify peaks on the chromatograms.

2.3. Sorbent preparation

Epoxysilica gel was prepared by modification of silica gel with γ-glycidyloxypropyltriethoxysilane in dry toluene according to a slightly modified procedure of Hermansson [19]. During silanization the reflux cooler was thermostated with water, the temperature being kept between the boiling points of methanol and toluene. After silanization for 8 h the epoxysilica was isolated by filtration, washed with toluene, acetone and methanol and dried at 110°C. The amount of bonded glycidyloxy groups was 3.2 μmol m^{-2} as calculated from elemental analysis data.

Diol-bonded silica gel was prepared from epoxysilica by opening the oxirane rings in an acidic environment. An aqueous suspension of epoxysilica was adjusted to pH 3 with 2% (v/v) nitric acid and stirred occasionally in an ultrasonic bath. About 10 min later the suspension was neutralized to pH 7 with sodium carbonate solution. The diol-silica obtained was filtered, washed with water and methanol and dried at 110°C.

To prepare dextran-grafted silica gels, dried epoxysilica gel was treated with aqueous solutions of dextran or AE-dextran at various pH values from 8 to 13.5. The volume of suspending solution was about twice the pore volume of added silica support. The suspension was stirred at 20°C for 48 h, then carefully adjusted to pH 3 with 2% (v/v) nitric acid. After several minutes the acidic solution was removed by washing the sorbent with water on a suction filter until the pH was 7. Finally, the dextran-grafted silica obtained was washed with methanol and dried at 105°C.

The amount of total immobilized organic phase was controlled by elemental analysis of treated supports during (Fig. 1) and after the grafting procedure (Table 2). The individual fractions of the sorbents were neutralized and thoroughly washed with water and methanol before elemental analysis.

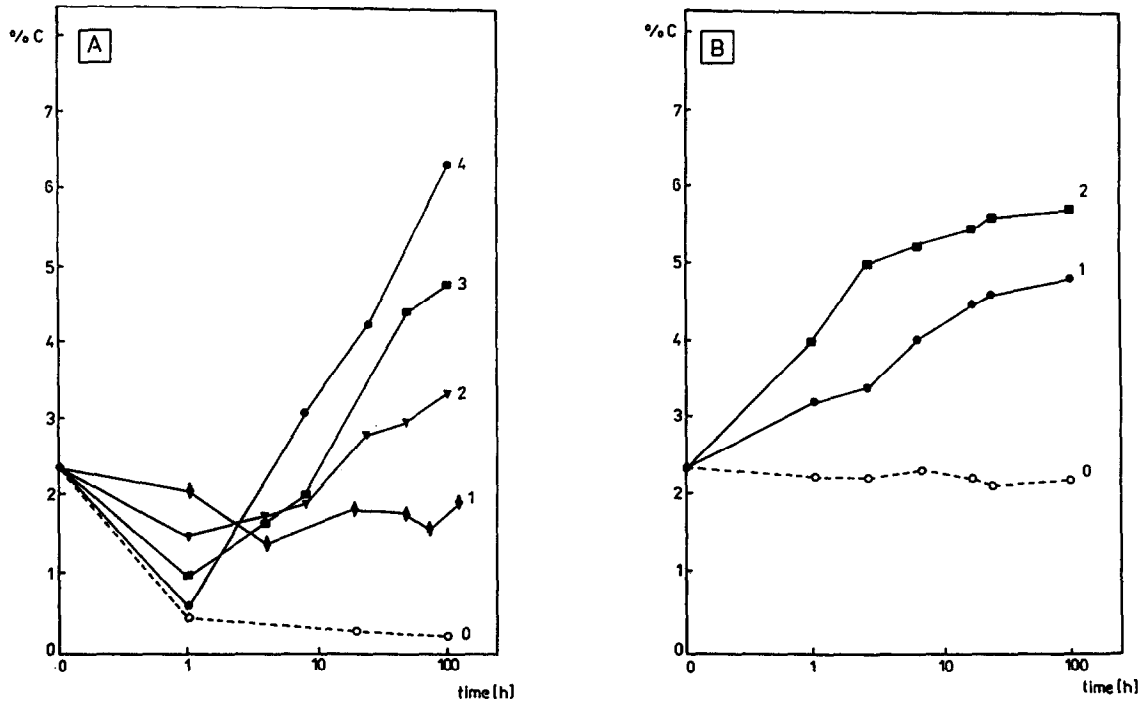

Fig. 1. Time dependence of total amount of immobilized organic phase, expressed as carbon percentage, during grafting dextran or its ethylenediamine derivatives on to epoxysilica gel. Content of dextran or its derivative in all grafting suspensions was 50% ($M_{dextran}/M_{support}$). Other conditions of reaction: (A) grafting native dextran at pH (1) 9.5, (2) 11.5, (3) 12.5 and (4) 13.5, and (0) reference experiment at pH 13.5 without dextran; (B) grafting ethylenediamine derivatives of dextran, (1) AE_3-D-40 and (2) AE_4-D-40, at pH 8.0, and (0) reference experiment at pH 8.0 without AE-dextran.

Table 2
Dextran-grafted silica gels

Sorbent	Immobilized dextran[a]	$\frac{M_{added\ dex.}}{M_{support}}$ [b]	$\frac{n_{NH_2}}{n_{AE\text{-}dextran}}$ [c]	pH at grafting	C (%)[d]
$SG\text{-}d_1$	D-40	1.0	–	13.5	6.4
$SG\text{-}d_2$	D-40	1.0	–	12.5	4.8
$SG\text{-}d_3$	D-40	1.0	–	11.5	3.4
$SG\text{-}d_4$	D-40	0.3	–	12.5	2.1
$SG\text{-}d_5$	D-40	0.3	–	9.5	2.0
$SG\text{-}d_6$	AE_1-D-40	0.5	0.2	11.5	2.2
$SG\text{-}d_7$	AE_2-D-40	0.5	1.0	11.5	4.0
$SG\text{-}d_8$	AE_3-D-40	0.5	2.2	8.0	4.8
$SG\text{-}d_9$	AE_4-D-40	0.5	5.7	8.0	5.8

[a] D-40 = Dextran with average molecular mass $40 \cdot 10^3$; AE-D-40 = Ethylenediamine derivative of D-40.
[b] Amount of dexran or dextran derivative in reaction mixture.
[c] Content of primary amino groups in the ethylenediamine derivatives of dextran used.
[d] As determined by elemental analysis.

2.4. Packing of columns

The sorbents were slurry packed into stainless-steel columns of dimensions 500 × 8 mm I.D., 250 × 8 mm I.D. and 100 × 4 mm I.D. using a Knauer (Berlin, Germany) pneumatic HPLC pump. To obtain well packed columns, the packing procedures needed to be optimized. We used either tetrachloroethylene–*n*-propanol [65:35 (v/v) before mixing] or toluene–cyclohexanol [40:60 (v/v) before mixing] as appropriate suspending liquids. As the pressing liquid we used methanol in both instances. The maximum packing pressure used was 30 MPa.

3. Results and discussion

3.1. Sorbent preparation

It was presumed that unmodified dextran reacts with oxirane groups bonded on the silica surface only in highly basic solutions (Fig. 1a). At pH 9.5 no important change in the carbon content of sorbent was observed, which indicates that virtually no dextran was immobilized under these conditions. At higher pH the unmodified dextran can be grafted on epoxysilica, but competition between cleavage of bonded glycidyloxypropyl groups and dextran grafting probably occurs. Hence it can be concluded that base-catalysed hydrolysis of Si–O–Si bonds prevails at the beginning but in the later stages of the reaction the silica surface is well shielded with grafted dextran. This prevents further splitting of residual silane-silica bonds and the grafting reaction prevails.

In the second approach the AE-dextrans with a controlled content of amino groups (Table 2) were grafted on epoxysilica at pH 8 (Fig. 1B). It seems that under these conditions the glycidyloxypropyl groups are not extensively cleaved. Therefore, the amount of grafted dextran could be approximately calculated from the difference between the carbon contents of the starting epoxybonded silica and the resulting dextran-grafted silica gel. For example, according to such a calculation, dextran-grafted silica composite sorbents SG-d_8 and SG-d_9 contain about 58.5 and 81 mg g^{-1} of immobilized dextran, respectively.

3.2. LC characterization of sorbents

Plots of the SEC distribution coefficient of the chromatographed proteins on the salt content in the mobile phase were used for the evaluation of both electrostatic and hydrophobic interactions of proteins with diol-bonded and dextran-grafted silica gels (Fig. 2).

The acidic protein ovalbumin and the basic protein chymotrypsinogen A were selected for a more detailed study of protein–sorbent interactions. These proteins have a relatively low molecular mass so that they can easily penetrate most pores of sorbents. The cation-exchange effect of residual silanols on both diol-bonded and dextran-grafted silicas increases the retention of chymotrypsinogen A, which is positively charged at pH 7. On the other hand, negatively charged ovalbumin is electrostatically repulsed and therefore it elutes from aqueous eluents with low ionic strength in a lower elution volume than predicted from the SEC calibration graph obtained under conditions of supressed solute–sorbent interactions (see Section 3.3). Both the retaining and repulsing electrostatic interactions can be easily supressed by adding salt to the eluent.

As shown in Fig. 2A, more effective shielding with the dextran layer may result in weakening of undesirable interactions of proteins with the silanols of the silica matrix as compared with diol-silica.

The effect of residual silanols is more effectively supressed by modification of epoxysilica gels with AE-dextran derivatives. The properties of the resulting sorbents depend, however, on the content of AE groups (Table 2) bonded on dextran prior its grafting on epoxysilica gel (Fig. 2B). Such a phenomenon is due to the prevention of the cation-exchange effect of silanols in the presence of positively charged amino groups. The optimum content of AE groups introduced on dextran is between 2 and 6 groups per

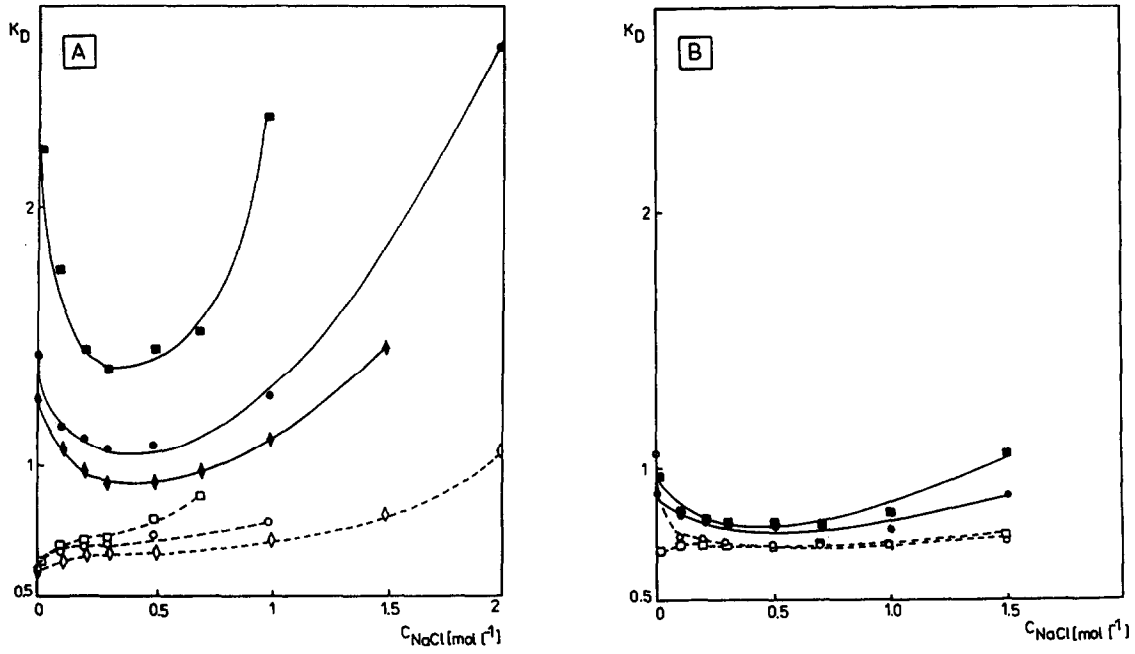

Fig. 2. Dependence of retention of ovalbumin (dashed lines) and chymotrypsinogen (solid lines) on salt concentration in the eluent obtained with various column packings. Eluent: 1/15 *M* phosphate buffer (pH 7.0) + NaCl additive. Retention was evaluated as SEC distribution coefficient $K_D = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume of the protein, V_0 the void volume measured as the elution volume of dextran with molecular mass $2000 \cdot 10^3$ and V_t the total liquid volume in the column measured as the elution volume of deuterium oxide. Column packings: (A) ■, □ = diol-SG, ◆, ◇ = SG-d_2; ●, ○ = SG-d_6; (B) ■, □ = SG-d_8, ●, ○ = SG-d_9.

dextran molecule. The dextran-grafted silicas designated SG-d_8 and SG-d_9 in Table 2 show lower protein–sorbent interactions than other dextran-grafted silicas. In this respect, the resulting materials show properties comparable to those of some well known commercial silica-based column packings for protein SEC such as TSK SW sorbents [13]. If the content of AE groups in the grafted dextran is higher than a certain level, anion-exchange properties of such materials can be observed. These result in increasing ovalbumin retention at low salt content in the eluent.

The hydrophobic interactions between the sorbent and proteins, caused by the hydrocarbon part of the glycidyloxypropylsilane spacer, begin to prevail at salt concentrations in the buffer of about 1 mol l^{-1} and higher. It can be seen from Fig. 2 that in this area the protein retention increases with increasing salt content more steeply with diol-silica than with dextran-grafted silicas. This means that also the protein–sorbent hydrophobic interactions can be significantly supressed by a dextran covering. This may be important for separation of hydrophobic proteins, such as membrane proteins.

In Fig. 3, plots of ln k' *vs.* the number of carbon atoms in *n*-alkanols chromatographed on diol-silica and selected dextran-grafted silica gel with water as eluent are compared. The lower selectivity of the separation of *n*-alkanols on dextran-silica under these reversed-phase conditions indicates its higher hydrophilicity in comparison with diol-silica prepared from the same starting epoxysilica. The average values of the selectivities obtained for adjacent pairs of *n*-alkanol homologues were 1.61 for diol-silica and 1.12 for dextran-grafted silica. The hydrophobic selectivities for *n*-alkanols of about 1.6 are typical of the hydrophilic brush-type silica-based

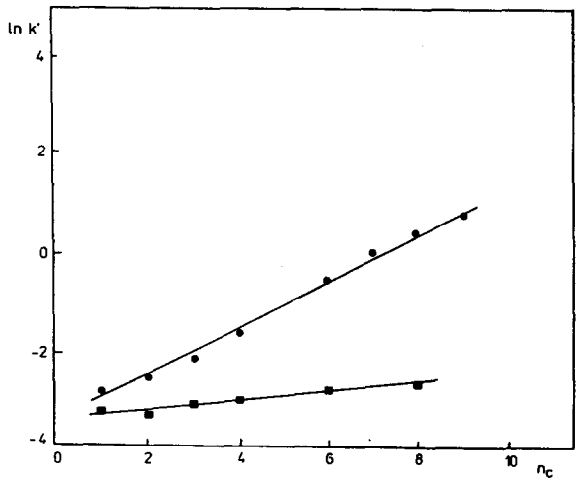

Fig. 3. Dependence of ln k' on carbon number of injected n-alkanols obtained with hydrophilic silica-based sorbents. ■ = Dextran-grafted silica SG-d_7; ● = diol-silica prepared from the same starting epoxysilica. Eluent: redistilled water.

phases for hydrophobic interaction chromatography of proteins and for aqueous SEC and the values well above 3 are typical of reversed-phase materials [20].

The preservation of the pore structure of both diol-silica and AE-dextran-grafted silicas was checked using the SEC calibration graphs for dextran standards (not shown). No significant differences between dextran-grafted, diol-bonded and bare silica gel were observed. Untypical shapes of the SEC calibration graphs were obtained for poly(ethylene oxides) in various eluents [21]. More precise measurements would be necessary to determine the degree of filling of the pore space by swollen dextran grafts.

3.3. Protein HPSEC

Dextran-grafted silica gels which show maximum hydrophilicity and minimum electrostatic interactions with proteins were packed into stainless-steel columns by the optimized slurry method. The protein SEC calibration graph (Fig. 4) obtained with the optimized dextran-grafted macroporous silica gel column is linear over a wide range of relative molecular masses (10^4–10^6) and show virtually no deviation from

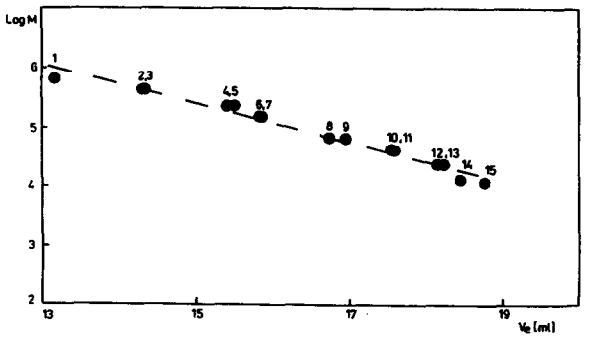

Fig. 4. SEC calibration graph for proteins obtained with a 25 × 0.8 cm I.D. column packed with SG-d_8 sorbent. 1 = TG; 2 = FR-B; 3 = FR-P; 4 = CAT-P; 5 = CAT-B; 6 = ALDO-P; 7 = ALDO-B; 8 = BSA-B; 9 = BSA-P; 10 = OVA-B; 11 = OVA-P; 12 = CHT-B; 13 = CHT-P and CHT-S; 14 = RN; 15 = CYT (abbreviations according the Table 1). Eluents, 1/15 M phosphate buffer (pH 7.0) containing 0.25 mol l^{-1} NaCl; flow-rate 2.0 ml min^{-1} detection at 289 nm.

linearity for both the acidic and basic proteins used in this study.

Fig. 5 shows an example of the SEC separation of a model mixture of proteins to demonstrate the resolving ability of such materials. It seems that the separation performance of the dextran-grafted silica columns is comparable to those obtained with commercially available silica-based stationary phases for protein HPSEC packed in columns of about the same size.

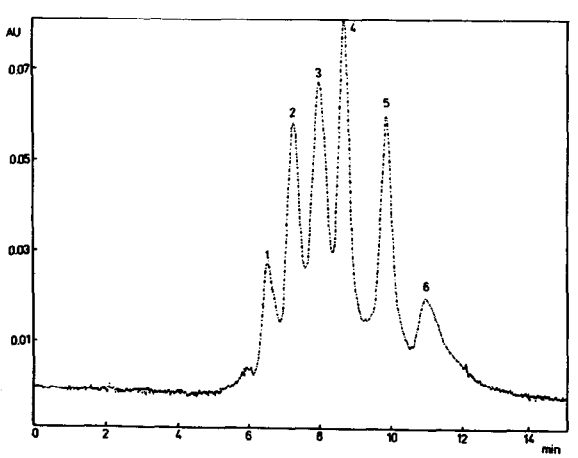

Fig. 5. HPSEC of standard protein mixture on a 50 × 0.8 cm I.D. column packed with SG-d_8 sorbent. Peaks: 1 = TG; 2 = FR-B; 3 = CAT-P; 4 = OVA-P; 5 = CYT-B; 6 = D,L-alanine. Detection at 230 nm; other conditions as in Fig. 4.

The linearity of the protein calibration graphs indicates the suitability of optimized dextran-grafted silicas for the determination of the molecular masses of proteins under appropriate operating conditions. For example, an alkaline proteinase sample obtained form a crude preparation of *Bacillus subtilis* proteinase [22] and soluble antigen from *Coxiella burnetti* propagated in chick embryo [23] were characterized. In both instances good agreement between the relative molecular masses found by polyacrylamide gel electrophoresis ($29.7 \cdot 10^3$ and $17 \cdot 10^3$, respectively) [22,23] and by HPSEC using dextran-grafted silica ($28 \cdot 10^3$ and $18 \cdot 10^3$, respectively) was observed.

4. Conclusions

An effective packing material for aqueous HPSEC was prepared by grafting dextran on to a macroporous silica support. The excellent mechanical stability of silica gel is maintained when the grafting procedure presented is applied. The electrostatic interactions of proteins with dextran-grafted silicas were minimized by combination of three effects: partial removal of silanols by reaction with γ-glycidyloxypropyltrimethoxysilane, steric shielding of residual silanols with grafted dextran macromolecules and electrostatic compensation of SiO^- groups by positively charged moieties introduced on to the dextran macromolecules prior to their immobilization. By grafting dextran on to epoxysilica, many hydroxyl groups are added to the sorbent surface. As a result, the sorbent hydrophilicity is enhanced and undesirable hydrophobic interactions with proteins are minimized. The optimized procedure for the synthesis of dextran-grafted silicas allows the preparation of sorbents showing minimum undesirable interactions with chromatographed proteins over a wide operating range of ionic strengths of the eluent. Well packed dextran-grafted silica columns with linear SEC calibration graphs for proteins have been successfully used as a medium for the HPSEC separation and characterization of proteins.

Dextran-modified solid supports may be successfully used not only in aqueous SEC but also, after additional modification, in ion-exchange [7] or affinity [9,24] chromatography of proteins.

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