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DEXTRAN-COATED SILICA PACKINGS FOR HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY OF PROTEINS

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SUMMARY

Porous silica beads have excellent mechanical properties for the high-performance liquid chromatography of proteins. However, the importance of non-specific interactions between silanols on the silica surface and the proteins requires a modification of these supports before they can be used as stationary phases for size-exclusion chromatography (SEC). Silica beads were coated with dextran bearing a small number of positive charges in order to neutralize the negatively charged silanol groups. Diethylaminoethyl-dextrans (DEAE-dextrans) with a relatively low percentage of dextran units bearing DEAE functions were layered on the silica beads. The influence of several characteristics of these packings on chromatographic performance were studied in order to determine the optimal conditions for the SEC of proteins.

INTRODUCTION

High-performance aqueous size-exclusion chromatography (HPSEC) is one of the most powerful techniques for the analysis and separation of biological macromolecules, especially proteins[1–4]. Columns packed with hydrophilic polymer gels[5,6], diol-bonded silica[7–10] and hydrophilic grafted[11] or coated[12] silica gels are currently used for the HPSEC of proteins in aqueous solution. One of the main requirements for an efficient high-performance liquid chromatographic (HPLC) support is mechanical stability of the matrix. In this respect, silica represents a good candidate as a starting material. However, the use of silica beads in the native form is not realistic, owing to its sensitivity to alkalis and the high level of non-specific interactions between silanol groups and proteins. Moreover, the commercially available grafted silica packings are expensive, limiting their use in preparative chromatography.

In this work, silica beads were coated with dextran bearing a small number of positive charges, which can neutralize the negatively charged silanol groups. Diethylaminoethyl (DEAE)-dextran with a relatively low percentage of dextran units bearing DEAE groups was layered on the silica beads. The different packings were found to have a minimal ion-exchange capacity. These beads possess high hydrophilicity, good porosity and resistance to alkalis and have lost their non-specific adsorption properties. The influence of several characteristics of these dextran silica packings (*e.g.*,

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porosity of starting silica beads, molecular weight of dextran, percentage of DEAE units, amount of polymeric coverage) on their performances in HPSEC was studied.

EXPERIMENTAL

Preparation of dextran-coated silica beads

Spherodex silica beads, provided by IBF Biotechnics (Villeneuve La Garenne, France), were porous and mainly spherical (40–100 μm). Before coating, the average pore diameter of the two types of silica beads were 125 and 30 nm, corresponding to specific surface areas of 25 and 125 m^2/g, respectively. They are generally used for preparative ion-exchange chromatography[13]. A solution of 40 g (1 mol) of sodium hydroxide in 80 ml of doubly distilled water was stirred with 20 g (0.122 mol) of dextran (T40, Batch No. KF 38205; T70, Batch No. LL01275; T500, Batch No. LL01035) provided by Pharmacia-France (Bois d'Arcy, France) in 80 ml of water for 30 min at 4°C. Then, 21 g (0.122 mol) of 2-diethylaminoethyl chloride hydrochloride (Janssen Chemica, Pantin, France) were added in several portions. The mixture was stirred at 4°C for 10 min and then heated at 60°C for 15–60 min. After the reaction, the mixture was cooled in an ice-bath and then adjusted to pH 9.0 using concentrated hydrochloric acid. DEAE-dextran was precipitated with methanol and the suspension was filtered with gentle suction in a 4-in. coarse sintered funnel[14]. Finally, the filter cake was washed with ethanol, which was drawn off through the filter. The partially dried product was ground to a powder and the remaining alcohol was removed under vacuum at 40°C. The characteristics of DEAE-dextrans were determined by titration and by elemental analysis. The percentage of dextrans units carrying DEAE groups varied from 0.9 to 24%.

A batch method followed by a cross-linking reaction was used in order to coat silica beads with DEAE-dextran[14]. A 10-g amount of silica was impregnated with 20 ml of modified dextran solution (8 g of DEAE-dextran in 100 ml) adjusted to pH 11.5. The packing was dried for 15 h at 80°C and the resulting powder was sieved. Then, 71 mg (0.55 mmol) of 1,4-butanediol diglycidyl ether (BDGE) (cross-linking agent) were added in 20 ml of a 0.3% (v/v) BDGE solution in diethyl ether for 10 g of silica. The mixture was stirred for 1 h at 40°C. After evaporation of the solvent, the silica powder was dried for 15 h at 80°C and the product was sieved. The amount of polymeric coverage, expressed as the weight percentage of carbon per gram of silica support, determined by elemental analysis, varied from 0.9 to 5%.

Chromatographic system

The chromatographic system we used was a Waters 510 HPLC pump from Millipore-Water France (Saint-Quentin Yvel, France) connected to a Rheodyne 7125 injection valve and to the column. A Uvicord-S UV detector, supplied by LKB (Orsay, France), was connected to a Hitachi D-2000 integrator (Laboratoires Merck-Clevenot, Nogent sur Marne, France).

The coated silica beads were introduced into a 25 × 0.4 cm I.D. stainless-steel column (Laboratoires Merck-Clevenot) by a dry packing method. The columns were generally eluted at a flow-rate of 2 ml/min with 0.05 *M* Tris–HCl–0.15 *M* sodium chloride (pH 7.4) buffer solution. The eluents were prepared with doubly distilled water and analytical-reagent grade reagents. The solutions were filtered through a

TABLE I

MOLECULAR WEIGHT MARKERS

Type	*Compound*	*MW (kilodaltons)*	*I**
Proteins	Thyroglobulin	669	
	β-Amylase	200	
	Alcohol dehydrogenase	150	
	Human transferrin	80	
	Bovine serum albumin	66	
	Pepsin	35.5	
	Human thrombin	33	
	Carbonic anhydrase	27.5	
	Trypsin	21.5	
	Myoglobin	17	
	Ribonuclease	13.7	
	Cytochrome *c*	12.4	
Pullulans	P1	853	1.14
	P2	380	1.12
	P3	186	1.13
	P4	100	1.10
	P5	48	1.09
	P6	23.7	1.07
	P7	12.2	1.06
	P8	5.8	1.07
	Cyclodextrin	1.135	

* $I = M_w/M_n$ = polydispersity index.

0.22-μm Millipore GS membrane and degassed. In order to establish calibration graphs on the different stationnary phases, 0.1 ml of a protein standard solution in the same buffer (2 mg/ml) (Sigma, St. Louis, MO, U.S.A.) was injected. Similarly, 0.1 ml of polysaccharide standard solution was also injected. These pullulans or polymaltotriose standards (Polymer Labs., Church Stretton, U.K.) have a narrow molecular weight distribution measured by the polydispersity index $I \approx 1$ (Table I). The elution properties of these compounds are determined by their elution volume, v_e, or their partition coefficient, k_D.

RESULTS AND DISCUSSION

The different modified silica columns were tested in HPSEC using several proteins with molecular weights from 6.5 to 700 kilodaltons. Blue dextran (2000 kilodaltons), glycylglycyltyrosinamide (434 daltons) and dinitrophenylalanine (255 daltons) were used in order to determine the exclusion limits of the stationary phase. Most of the proteins were eluted between the the exclusion limits of the stationary phase, except cytochrome *c*, which was abnormally retained by the modified silica. This effect is probably due to the high p*I* value and to the small size of this protein and indicates that some silanol functions inside the pores are not completely masked by the DEAE-dextran.

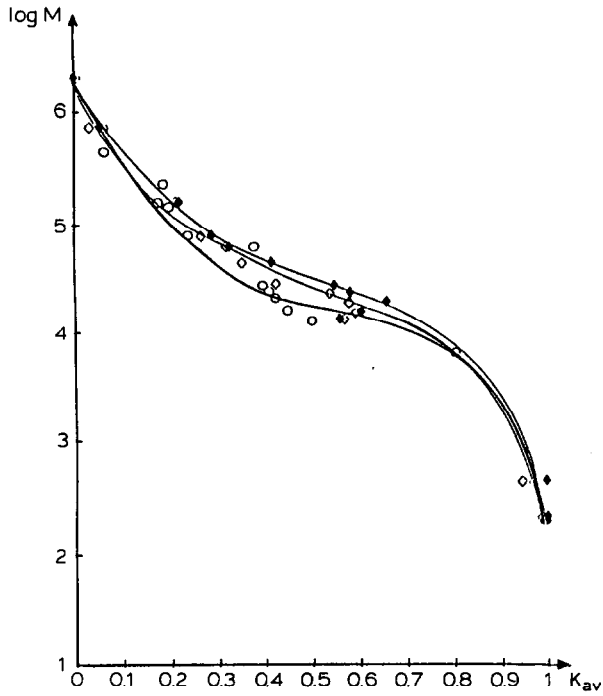

Fig. 1. Molecular-weight calibration graphs for proteins with different dextran-coated silica supports. Column, 25 × 0.7 cm I.D.; flow-rate, 2 ml/min; buffer, 0.05 *M* Tris–HCl–0.15 *M* NaCl (pH 7.4).

	DEAE (%)	*C (%)*	*Dextran MW (kilodaltons)*
SIDDEX X0150701 (○)	13	4.7	70
SIDDEX X0150403 (◇)	7	3.8	40
SIDDEX X0155001 (◆)	4	4.6	500

The influence of the molecular weight of the coating dextran polymers used to coat the 125-nm pore size silica on the elution of proteins is shown in Fig. 1. Three DEAE-dextrans were tested with different percentages of dextran units bearing DEAE groups (from 3.8% to 4.7% of carbon) of molecular weight 500, 70 and 40 kilodaltons. The calibration graphs are very similar for these three SIDDEX packings. This result indicates that the molecular weight of the DEAE-dextran has no influence on the neutralization effect. In fact, the size of the different modified dextran polymers is relatively low compared with the mean value of the pore diameters of the native silica. This allows good penetration of all hydrophilic polymers, including high-molecular-weight samples (500 kilodaltons).

DEAE-dextrans with different molecular weights and different DEAE ratios exhibited similar chromatographic properties. In fact, the amount of polymeric coverage is more important if the percentage of dextran unit bearing DEAE groups is in the range 4–13%. Below 4%, the neutralization of negative charges is not completely achieved. In contrast, above 10%, an excess of positive charges is present on the coated surface, which interferes with the steric exclusion mechanism. Such packings exhibit cation-exchange properties.

A similar behaviour was observed on the 30-nm pore-size silica supports (Fig.

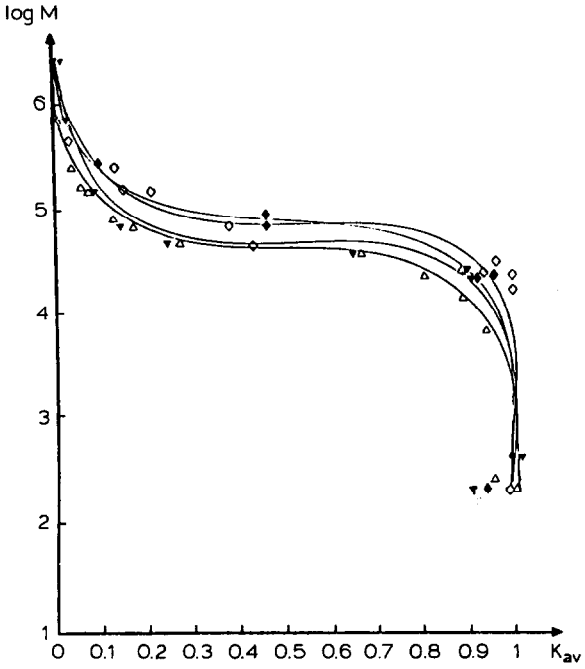

Fig. 2. Molecular-weight calibration graphs for proteins with different dextran-coated silica supports. For elution conditions, see Fig. 1.

	DEAE (%)	*C (%)*	*Dextran MW (kilodaltons)*
SIDDEX X0750701 (◇)	13	4.6	70
SIDDEX X0750702 (▼)	4.3	2.57	70
SIDDEX X0750704 (△)	4.45	4.84	70
SIDDEX X0750404 (◆)	5	2	40

2). In the same way, the molecular weight and the percentage of units bearing DEAE groups do not notably affect the separation properties of the phases. A value of 4–13% is necessary to obtain a good polymer coverage.

A comparison of the behaviours of the two types of silica coated with the same DEAE-dextran is illustrated in Fig. 3. The slopes of the calibration graphs indicate that the support prepared with 30-nm silica beads yields a better resolution for proteins in the range 10–100 kilodaltons. However, the molecular weight limits of this support are slightly restricted compared with the exclusion limits of a 125-nm pore-size unmodified silica packing.

Finally, the influence of the flow-rate was examined with different coated silicas. A good separation is still observed with a high flow-rate (1.5 ml/min) but the resolution decreases. However, such supports used in our analytical chromatographic system cannot give high resolutions. In fact, the diameter of the beads (40–100 μm) is too large in relation to the dimensions of the analytical column. This type of silica should be used for scaling up chromatographic separations of proteins.

A comparison of the calibration graphs of proteins and polysaccharide standards (Fig. 4) indicates that these two types of water-soluble polymers are normally eluted between the exclusion limits of the SIDDEX supports. The differences ob-

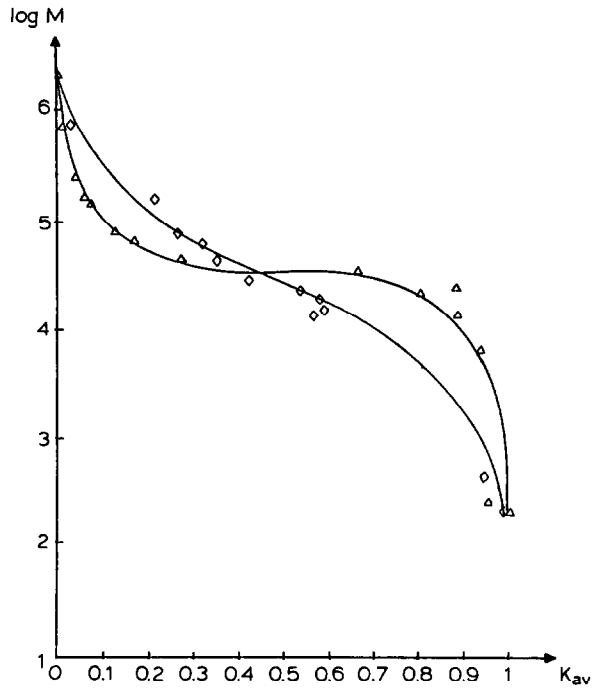

Fig. 3. Molecular-weight calibration graphs for proteins with different dextran-coated silica supports. For elution conditions, see Fig. 1.

	Average pore diameter (nm)	*DEAE (%)*	*C (%)*
SIDDEX X0750704 (△)	125	4.45	4.84
SIDDEX X0150403 (□)	30	7	3.8

served in the slopes and the exclusion limits are related to the different structural forms of these two types of macromolecular compounds in the aqueous elution buffer[15].

Consequently, the optimal conditions for efficient SEC of proteins on such coated silica supports are the following: (1) 70-kilodalton molecular weight of starting dextran; (2) 5% of dextran units bearing DEAE groups; (3) 4–10 g of carbon per 100 g of support as polymer coverage, corresponding to 0.1035 g/m^2 of dextran; (4) flow-rate 1 ml/min; and (5) eluent 0.05 *M* Tris–HCl (pH 7.4)–0.15 *M* NaCl.

CONCLUSION

Aqueous SEC of proteins can be achieved on silica beads after a preliminary neutralization of the negatively charged silanol groups. Silica beads can be neutralized by coating with dextrans bearing a small number of positively charged DEAE units. These DEAE units produce a polymeric layer of the hydrophilic dextrans on the silica surface. In addition, the DEAE units neutralize the ion-exchange capacity of native silica. In order to avoid the leakage of coating polymer, the DEAE-dextrans covering the silica beads are cross-linked. Hence the interactions between proteins in

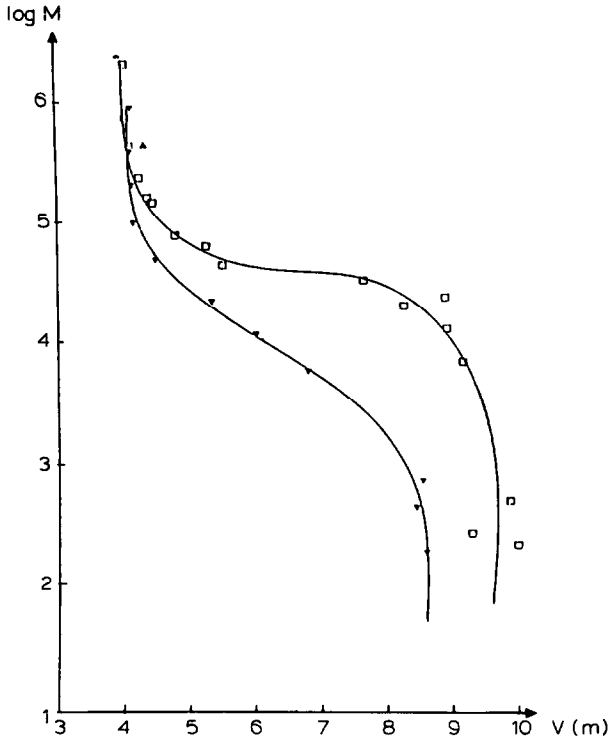

Fig. 4. Molecular-weight calibration graphs for proteins (□) and polysaccharide standards (▼). For elution conditions, see Fig. 1.

solution and the silica supports are minimized and the efficiency of such stationary phases in HPSEC is improved.

This study demonstrates that the amount of polymer coverage is the most important parameter for good neutralization. A minimum of 4% of dextran units bearing DEAE groups on the hydrophilic polymers covering the silica surface is necessary to minimize the ion-exchange capacity of the silica. Silica beads coated with DEAE-dextrans possess good hydrophilicity and an excellent chemical resistance to alkalis while maintaining the mechanical properties of the inorganic starting material. If the percentage of dextrans units bearing DEAE groups exceeds 13%, the cation-exchange capacity of the supports interferes with the steric exclusion mechanism.

On these passivated silica supports, very fast separations of proteins can be achieved and easily scaled up. These modified silica supports can be used for the preparative HPSEC of biological products.

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