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A NEW, STABILIZED, HYDROPHILIC SILICA PACKING FOR THE HIGH-PERFORMANCE GEL CHROMATOGRAPHY OF MACROMOLECULES

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SUMMARY

A new approach for the stabilization of bonded-phase silica packings for the gel chromatography of proteins has been examined. Porous silica microspheres were stabilized by a zirconium salt treatment and then covalently bonded with a hydrophilic organo-silane. The resultant product (Zorbax® Bio Series GF-250) is a high-resolution, high-speed chromatographic packing with enhanced bonded-phase stability in aqueous buffered mobile phases. Packed columns of this material fractionate proteins roughly by molecular weight (MW) in the range of from 12 000 to 120 000 Daltons with approximately log MW–elution volume linearity. The zirconium-treated surface presents no unusual restraints in operating conditions and even permits short-term use of buffered eluents at pH 9. The useful operating lifetime of this packing has been shown to surpass some other currently available products.

INTRODUCTION

Gel chromatography[1] (or size-exclusion chromatography), described in detail by Yau *et al.*[2], is a highly useful technique for the characterization of organic polymers. Proteins, enzymes, and other macromolecular substances have also been separated on the basis of molecular size by this technique. Conventionally, highly cross-linked dextran gels are utilized for this application, because of their chemical inertness and low non-specific adsorption properties. However, such gels are not rigid, and thus swell considerably in aqueous buffers, and because they cannot be used at high flow-rates, long separation times often result.

Modern, small-particle packings, comprised of silica, cannot be directly substituted for dextran gels in an attempt to achieve speed and high resolution, because the native surface of silica readily adsorbs various proteins, and those which are not bound irreversibly are frequently denatured after elution. Therefore, in order to use silica packings for the chromatography of biological substances successfully, the silica surface must first be modified.

Several researchers[3–9] have used chemical bonding of organo-silanes to derivatize the silica surface. This method deactivates the surface and provides the chemical inertness needed for proteins while producing the resolution which is the object of

the use of small-particle packings. Adsorption effects are minimal, and the recovery of biologically active materials is usually good.

Silica, glass, and other inorganic support matrices used in gel chromatography are soluble to some extent in buffered mobile phases. This dissolution phenomenon is a function of hydroxide ion concentration and, at levels higher than neutral pH, reduces the useful operating lifetime of the column significantly. The bonded phase is hydrolyzed continuously and voids are commonly observed in the packed bed.

Tomb and Weetall[10] and later Marsh and Tsao[11] employed a coating technique using zirconium salts to deposit a continuous layer of metal oxide on the surface of porous glass. The object was to improve the water durability of the support. Subsequent covalent attachment of first organo-silanes and then glucoamylase produced an improved immobilized enzyme composite for use in packed bed enzyme reactors. This technique was extended to produce chromatographic supports for gel chromatography and marketed by Pierce Chemical Company. This product is no longer commercially available.

Although the details of the stability of glass and siliceous based packings for gel chromatography are not adequately described in the literature, manufacturers of such products are certainly aware of these problems. Some suppliers, such as Toyo Soda[12], have employed polymers to help stabilize their packing materials. However, even polymer-coated silica packings exhibit this dissolution problem since the base can eventually penetrate the silica surface below the polymer allowing water soluble organo-silicates to form.

A different approach, useful for stabilizing organo-silane–silica against hydrolytic attack, is the subject of this paper. This method is based on the hypothesis that coating the entire surface of silica is neither necessary nor desirable. A metal oxide would be utilized to partially modify the surface making it less sensitive to hydrolysis. This could be followed by deactivating the surface by molecular monolayer bonding with a hydrophilic organo-silane. The resultant packing material would then have increased protection against dissolution of silica and also possesses the inert hydrophilic surface character for the separation of proteins and other biological substances. The partial coverage of the surface with metal oxide, different from previous methods[10,11], should preserve the pore volume which is essential to resolution in this chromatographic mode[2]. Polymeric bonded phases, which also decrease pore volume, and are used to enhance stability, should no longer be necessary. The results of such an investigation are described in this paper.

EXPERIMENTAL

Chromatographic equipment

A Du Pont Model 8800 HPLC system (Du Pont Company, Wilmington, DE, U.S.A.) was employed, consisting of a pump, oven compartment, gradient controller, injection valve (Rheodyne Model 7120), and UV spectrophotometer, set at 225 nm. The detector output was interfaced to a DEC 10 (Digital Equipment Corp., Maynard, MA, U.S.A.) data handling system[13] for data processing and calculation of chromatographic parameters.

Chemicals and reagents

Sulfamic acid, ammonium sulfate, sodium chloride, sodium phosphate, and sodium azide were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and used without further purification. Tris was obtained from Sigma (St. Louis, MO, U.S.A.). Water was distilled and deionized. Zirconium oxychloride was purchased from Alfa Products (Division of Thiokol, Danvers, MA, U.S.A.). Tetrahydrofuran (THF) was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Freon® TF was purchased from Du Pont (Wilmington, DE, U.S.A.). Glycidoxypropyltrimethoxy silane (G6720) was obtained from Petrarch Systems (Levittown, PA, U.S.A.).

Table I gives a list of the proteins used in the experiments. These substances were used as received. The samples were gently dissolved in the mobile phase at a concentration of 1 mg/ml.

Mobile phase

Buffer solutions were prepared in four batches and were comprised of 0.2 *M* ammonium sulfate or sodium chloride, 0.05 *M* Tris or dibasic sodium phosphate, and 0.001% (w/w) sodium azide. The pH of the solutions was adjusted with sulfamic acid. For buffers of higher pH sodium borate was used.

In initial experiments, the mobile phases were passed through the column once, then discarded. The longer-term column stability studies consumed considerable mobile phase when used this way. Therefore, duplicate experiments were carried out in which a single four-batch of buffer was continuously recycled. These studies indicated no difference between using recycled or fresh mobile phase. Recycling of the mobile phase was used for the majority of the long-term stability studies reported here. A 50 × 4.6 mm I.D. pre-column, packed with 18 μm, 60 Å, Zorbax® ODS (Du Pont) was inserted between the pump and sample injection valve to remove particulates and dissolved substances in the recycled mobile phase, which may foul the chromatographic column.

Column packing materials

Zorbax® PSM (porous silica microspheres) 150 (particle size, 4.0 μm, average B.E.T. pore size, 167 Å, B.E.T. specific surface area, 144 m^2/g) was produced by

TABLE I

PHYSICAL PARAMETERS OF PROTEINS

Protein	*MW (Daltons)*	*Isoelectric point*	*Source**
Thyroglobulin	660 000	–	A
Ferritin (equine)	440 000	–	B
Immunoglobulin (Cohn II fraction)	160 000	*ca.* 7.7	A
Bovine serum albumin	69 000	4.98	A
Ovalbumin (egg)	43 500	4.7	A
β-Lactoglobulin (bovine)	36 000	5.1	A
Myoglobin (whale)	17 500	6.9	B
Lysozyme (egg)	14 100	11.00	B
Ribonuclease (bovine)	13 500	8.7	A
Cytochrome *c*	12 200	9.0	A

* A = Sigma (St. Louis, MO, U.S.A.) and B = Calbiochem-Behring (LaJolla, CA, U.S.A.).

using the procedures of Iler and McQueston[14]. Toyo Soda TSK G3000SW columns of 300 × 7.5 mm I.D. were purchased from Bio-Rad Labs., (Richmond, CA, U.S.A.) under the tradename Bio-Sil® TSK.

Stabilized packing preparation

The PSM 150 silica packing was refluxed for 30 min in an aqueous 1% (w/v) zirconium oxychloride solution. The slurry was cooled, filtered, then dried in a 100°C vacuum oven. The zirconium-treated silica was then fired in an electric furnace at a temperature above 800°C for 18 h. The DIOL (di-alcohol hydrophylic functional group)-bonded phase was grafted to the zirconium-modified silica surface by refluxing the packing in a 10% (v/v) solution of glycidoxypropyltrimethoxysilane in 0.01 *M* dibasic sodium phosphate (pH 8.8) for 16–18 h. A control batch of non-stabilized DIOL packing was prepared by the method given by Yau *et al.*[2] but the procedure was modified by using 0.01 *M* monobasic sodium phosphate (pH 3.5).

These stabilized, hydrophilic, bonded-phase packings were high-pressure slurry packed into 250-mm long columns of 6.2 mm or 9.4 mm I.D. by means of Du Pont proprietary column loading procedures. Other column loading techniques, such as those described by Snyder and Kirkland[15], should also be suitable for preparing similar columns.

RESULTS AND DISCUSSION

Zirconium treatment

The levels of zirconium on the treated silica surface are 1–2% (w/w), as measured by atomic absorption and inductively coupled plasma atomic emission spectroscopy techniques (ICP-AES). These levels are too low (*ca.* 1–1.5 μmoles/m²) for complete coverage of the silica surface with zirconium. Efforts to determine the distribution of the zirconium atoms on the surface of the matrix employing such techniques as electron spectroscopy for chemical analysis (ESCA), diffuse reflectance Fourier transform infrared spectroscopy (DRIFT), and X-ray diffraction were unsuccessful, owing to the low levels of zirconium. However, some information was derived from ^{29}Si-^{1}H nuclear magnetic resonance by cross-polarization and magic-angle spinning (CP-MAS)[16,17]. This technique allows the examination of three types of surface silicon atoms, which can be differentiated by their proton atomic environments:

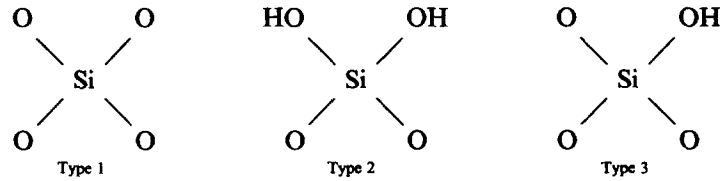

Fig. 1A shows the nuclear magnetic resonance (NMR) measurement of these three types of silicon atoms on the surface of an untreated sample of Zorbax® PSM 150 silica. Fig. 1B shows the effect on these surface species of the zirconium treatment described in the experimental section. The Type 2, geminal groups, are essentially gone and the Type 3 silanol groups are greatly diminished. It is interesting to note that treatment of the silica with a small-chain organo-silane (trimethylchlorosilane) also virtually eliminates these two species (Fig. 1C). While it is difficult to be very

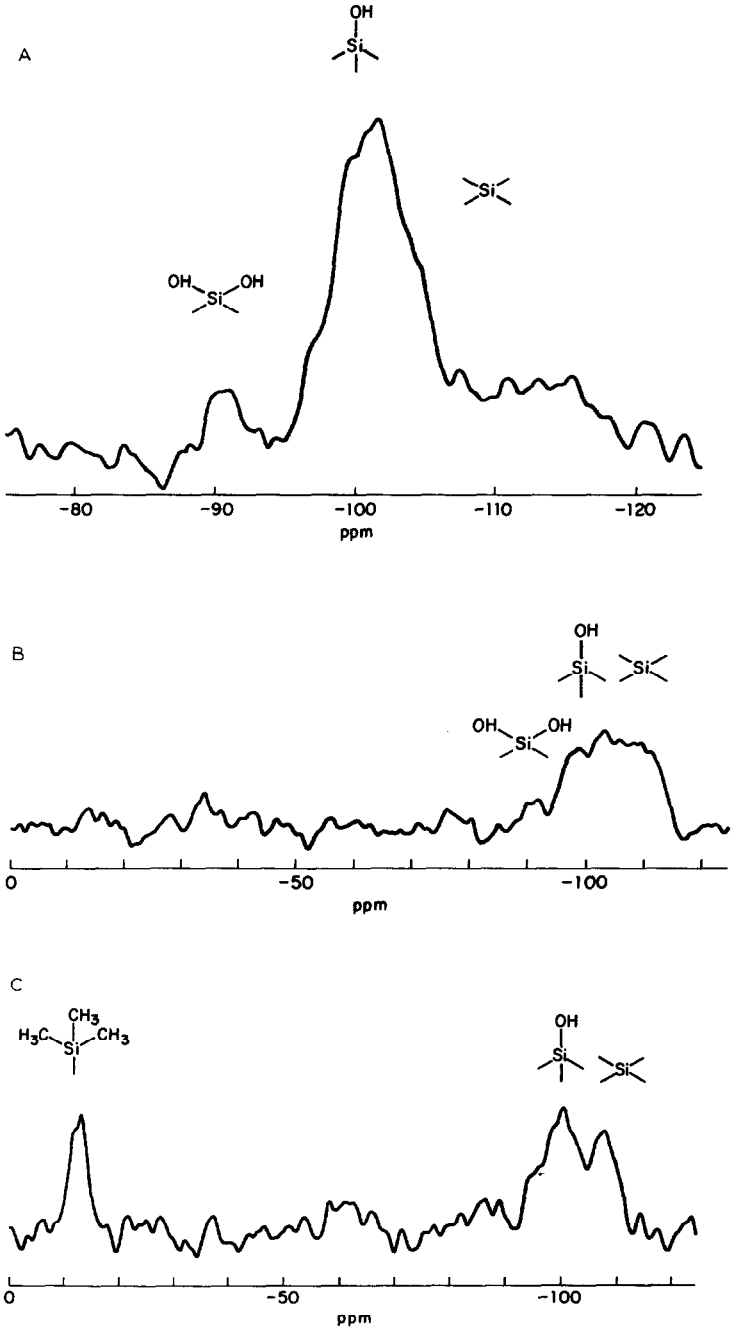

Fig. 1. A, ^{29}Si-^{1}H CP-MAS NMR spectrum of pure silica, Zorbax® PSM 150 SIL (untreated silica). Geminal silanol [$=Si(OH)_2$], -90.34 ppm; silanol [$=Si(OH)$], -100.5 ppm; substructural silica [$=Si=$], -115.3 ppm. B, Spectrum of zirconia stabilized PSM 150 silica. Same assignments as in A. C, Spectrum of Zorbax® PSM 150 SIL bonded with trimethylchlorosilane (TMS). Bonded phase [$-Si(CH_3)_3$], -13 ppm. Other assignments same as in A.

quantitative under these conditions of signal to noise ratio, it is clear that the zirconium must be interacting with the Type 2 and Type 3 silanol species, the same ones which are involved with the chemical bonding of organo-silanes. Thus, pre-treatment of the silica with the zirconium salt prior to the reaction with organo-silanes must change the chemical nature of the organo-silane–silica interface.

The effects of the zirconium treatment are apparently limited to the surface of the silica at the levels studied. The specific surface area of the silica packing is unchanged following the treatment. The pore structure is only slightly affected by the added zirconium and the monolayer organo-silane coating, as demonstrated by a size-exclusion separation of polystyrene standards (Fig. 2) with THF as the mobile phase. A plot of log molecular weight (MW) *versus* distribution coefficient(K_D) can be constructed from the data in Fig. 2 to produce the curves shown in Fig. 3. Pore volume is occupied by both the metal oxide and the organic bonded phase. Both factors contribute to only a small reduction in pore volume, the magnitude of which affects smaller molecules more than larger ones, as would be expected.

Fig. 3 also shows a calibration plot for proteins on the zirconium-treated, DIOL-coated material after flushing the column with water and equilibrating with buffer. The plot shows that these materials follow a different separation pattern from that of the polystyrene standards. This variance results from differences between the

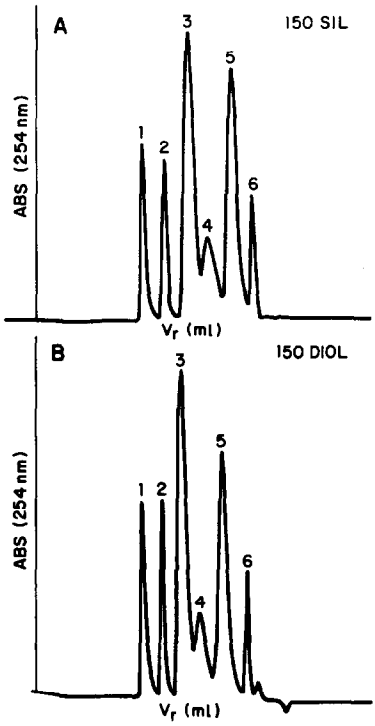

Fig. 2. A, Zorbax® PSM 150 SIL and B zirconium-stabilized GF-250 DIOL used in the size-exclusion mode with polystyrene (PS) molecular weight standards. Peak identities: (1) PS MW 1 800 000, (2) PS MW 50 000, (3) PS MW 17 500, (4) PS MW 9000, (5) PS MW 2000, (6) toluene MW 92. Mobile phase, THF. Flow-rate, 1.0 cm min^{-1}. Detector, UV absorbance, 0.64 a.u.f.s. (254 nm). Temperature, ambient.

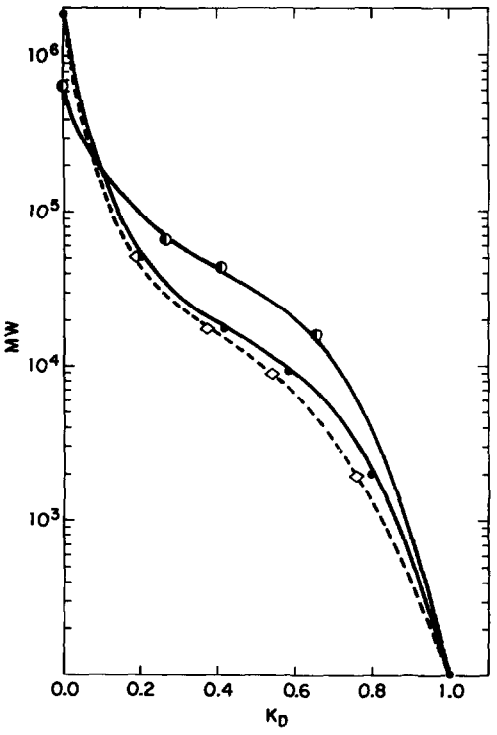

Fig. 3. K_D plot for polystyrene standards and proteins on PSM 150 SIL and zirconium-stabilized GF-250 DIOL. (●), PS standards for DIOL: (◇), PS standards for SIL: (◐), protein standards for DIOL. Data used to construct plots for polystyrenes are taken from Fig. 2. Data for proteins are taken from chromatograms generated under the same conditions as in Fig. 2 except mobile phase, 0.2 *M* sodium chloride, 0.01 *M* Tris, 0.005% sodium azide (pH 7.67); and detector, 225 nm.

two types of molecules (*i.e.* their hydrodynamic volume *versus* molecular-weight characteristics).

Stability tests

The stability of polar bonded phases has not been well studied. A realistic, yet sensitive test is required which allows the degradation to be followed. The test chosen for this study involved operating conditions for temperature, flow-rate, and buffer composition which were similar to those used in common practice. The pH of the mobile phase was adjusted to a higher-than-normal level (8.25 ± 0.05) in order to accelerate degradation of the columns[18]. Additional studies expanded the pH range to include levels from pH 3.0 to 9.44. Lysozyme was chosen as a convenient test material, since it is an enzyme which is quite basic and, therefore, sensitive to the presence of exposed, acidic silanol groups on the packings surface. The increase in retention volume (V_r) of lysozyme relative to the permeation volume (V_m) of the column is proportional to the loss of bonded phase by hydrolysis.

Fig. 4A and B show two protein separations on a fresh, stabilized DIOL column, following a short equilibration with buffered (pH 8.2) mobile phase. The number of column elution volumes ($V_{m,n}$) was approximately 10 ($V_{m,10}$). Fig. 7C and D

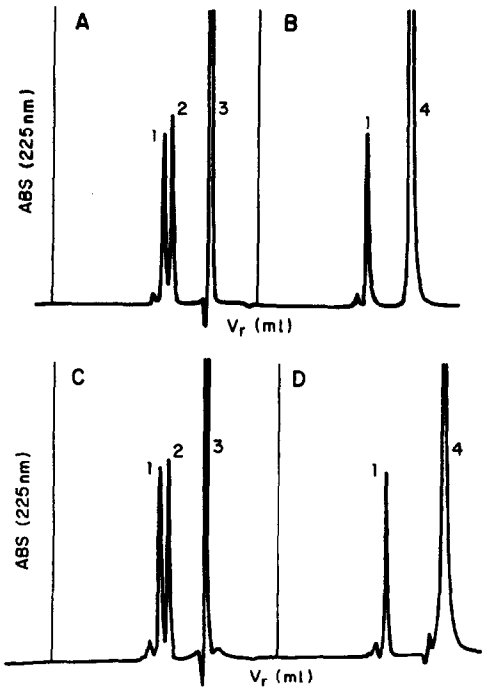

Fig. 4. Bonded-phase stability test. Chromatograms of zirconium-stabilized GF-250 DIOL, 250 × 6.2 mm I.D. A and B, Start of test ($V_{m,10}$); C and D, end of test ($V_{m,3500}$). Peak identities: (1) bovine serum albumin, (2) ovalbumin, (3) sodium azide, (4) lysozyme. mobile phase: 0.2 *M* ammonium sulfate, 0.05 *M* Tris, 0.005% sodium azide, pH 8.25. Flow-rate: 0.6 ml min^{-1}. Temperature ambient. Inlet pressure: 550 p.s.i.g. Detector: absorbance, 0.64 a.u.f.s. (225 nm).

show the same separations after 630 h of continuous elution. Approximately 3500 permeation volumes ($V_{m,3500}$), or 19 l of mobile phase, were eluted through this column prior to these experiments. Only slight degradation of this column can be observed as a small shift in the retention of lysozyme. Some band broadening of the lysozyme peak is also evident. It is interesting that no changes were observed in retention volume or bandwidth for proteins other than lysozyme.

The chromatograms in Fig. 5 show the results of a similar study with a fresh Toyo Soda TSK G3000SW column. The use of this type of column has been reported extensively in the literature for the separation of proteins[19–26]. The test was stopped after 192 h of continuous use of 9.8 l of mobile phase, ($V_{m,811}$), because significant degradation was observed. Following the chromatographic experiments shown in Fig. 5C, D, the column was opened and a 1-cm void in the packing was observed. Subsequent analysis of the packing also indicated that 37% of the bonded phase was lost.

The relative stabilities of these test columns are illustrated in Fig. 6, which shows a plot of the retention (k') of lysozyme against the number of permeation volumes of mobile phase passed through the test columns. Under these test conditions [0.2 *M* ammonium sulfate (pH 8.25 ± 0.05)], the zirconium-stabilized silica is shown to be significantly more stable than either the TSK column or a non-stabilized silica column.

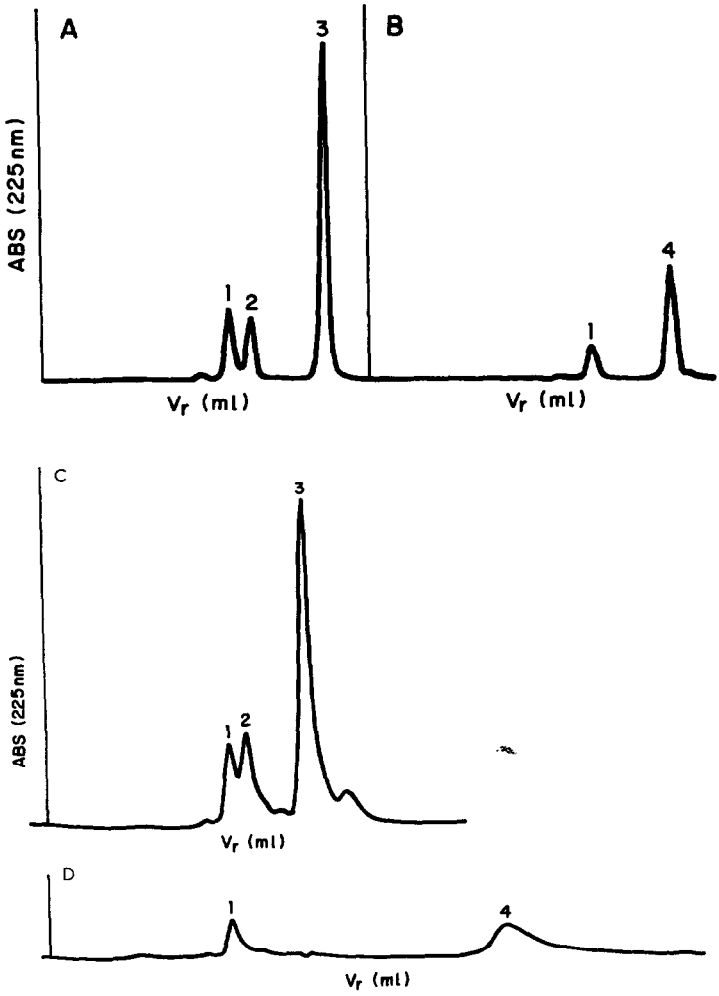

Fig. 5. Bonded-phase stability test. A and B, Start of test ($V_{m,10}$); C and D, end of test ($V_{m,811}$). Toyo Soda TSK G3000SW 300 × 7.5 mm I.D. Same conditions and peak identities as in Fig. 4, except flow-rate: 0.85 ml min^{-1}.

The pH of the mobile phase was varied from 3.0 to 9.2 in order to assess the stability of the zirconium-treated GF-250 DIOL columns as a function of the concentration of hydroxide ions. Fig. 7 shows the retention of lysozyme, plotted against the number of column permeation volumes for four pH values. As expected, the retention of lysozyme changes very little at pH 7.0, while more rapid degradation can be observed when the buffers are adjusted to higher pH levels.

Table II provides a summary of the results of these experiments, along with data on surface coverage. Seven columns were used in the stability experiments: five treated with zirconium, one non-stabilized, and a TSK G3000SW. An additional pristine TSK G3000SW column was sacrificed to obtain a comparison between the levels of carbon at the start and end of the experiment. The carbon content of our

TABLE II

SUMMARY OF STABILITY TEST RESULTS FOR ZIRCONIA STABILIZED GF-250 DIOL, NON-STABILIZED DIOL, AND TOYO SODA TSK® G3000SW DIOL COLUMNS

Column		*Start of test*		*End of test*		*Loss in bonded phase (%)*	*Test enzyme*		*Total volumes, V_T (l)*	*Number of permeation volumes ($V_{m,n}$)*	*pH of mobile phase*
		%C	*μmoles/m²*	*%C*	*μmoles/m²*		*k'_i*	*k'_f*			
GF-250 DIOL	1	3.96	4.12	3.72	3.87	6	0.001	−0.001	6.05	1172	3.0
	2	3.96	4.12	3.69	3.84	3	−0.05	0.077	19.0	3600	7.0
	3	3.96	4.12	3.61	3.75	9	−0.03	0.08	18.14	3509	8.25
	4	3.96	4.12	3.32	3.45	16	−0.023	0.513	22.03	4286	8.45
	5	3.96	4.12	3.35	3.49	16	0.23	0.44	4.9	979	9.2
	6*	3.19	3.32	2.19	2.28	31	0.00	2.76	0.8	138	8.25
TSK G3000SW	7	8.27	−**	5.25	−**	37	−0.06	0.762	9.8	811	8.25

* Non-surface stabilized.

** Polymer material. Formula weight unknown.

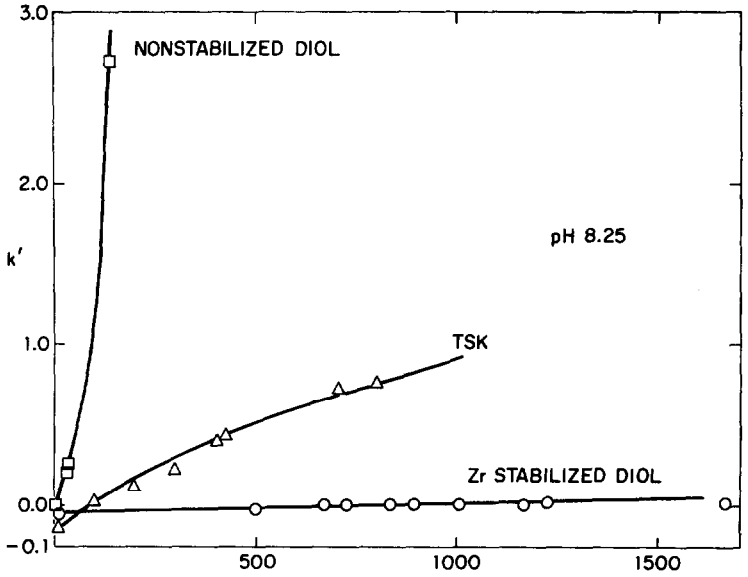

Fig. 6. Plot of k' of test enzyme (lysozyme) *versus* number of permeation volumes eluted, at pH 8.25. (○) zirconium-stabilized GF-250 DIOL: (△) Toyo Soda TSK G3000SW; (□) non-stabilized DIOL (no zirconium). Same operating conditions as in Fig. 4.

test packings indicated that initially there was a significant difference in surface coverage between the zirconium-treated and non-stabilized packings (*e.g.* 4.12 μmoles/m² *versus* 3.32 μmmoles/m²). These packings differ only in surface treatment and the bonding reaction pH as they were prepared from the same lot of silica. A non-stabilized version cannot be prepared using an otherwise identical bonding reaction due to harsh conditions (90°C, pH 8.8), hence, the pH used was 3.5. Another

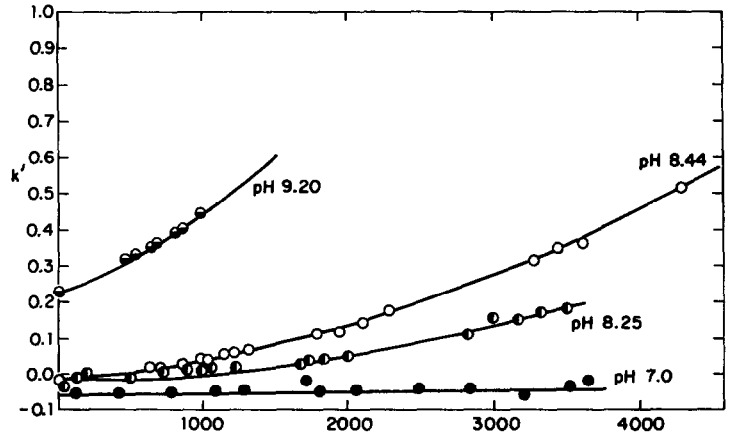

Fig. 7. Plot of k' of test enzyme (lysozyme) *versus* number of permeation volumes eluted at several pH values. pH 7.0; (●), pH 8.25; (◐), pH 8.44; (○), pH 9.20; (◒). Same operating conditions as in Fig. 4, except for pH; 0.2 *M* sodium borate buffer used for pH 9.20 mobile phase.

consideration is the dehydration of the silanols during the high-temperature metal oxide treatment procedure. The high pH of the aqueous reaction slurry regenerates the population of silanols as the bonding reaction proceeds.

The net loss in bonded phase, calculated from carbon content, %C, can be seen to vary from 3% to 16% as the pH changes from 3.0 to 9.2. The range between the initial capacity factor (k'_i) and final capacity factor (k'_f) for the test enzyme, lysozyme, can be seen to be roughly correlated with the loss in bonded phase. It is interesting to find that even after 16% of the bonded phase is lost (pH 8.25), the column still functions well. This observation may imply that there is some "extra" material present that is removed during use which is not essential for shielding the proteins from the silica surface. No kinetic experiments were performed to investigate this observation further. The Toyo Soda G3000SW column lost 37% of its bonded phase under these conditions, and unlike the zirconium-stabilized packed column, lost plate efficiency due to collapse of the packing material.

Chromatographic factors

Gel chromatography does not separate substances strictly by molecular weight but rather by an entropy-driven process, created by differences in the hydrodynamic volume occupied by molecules, as determined by their radii of gyration[2]. While proteins have definite structures, they are not spherical, strictly speaking, nor do they conform to rod-like or random-coil geometries. In addition, other mechanisms[3–9] also

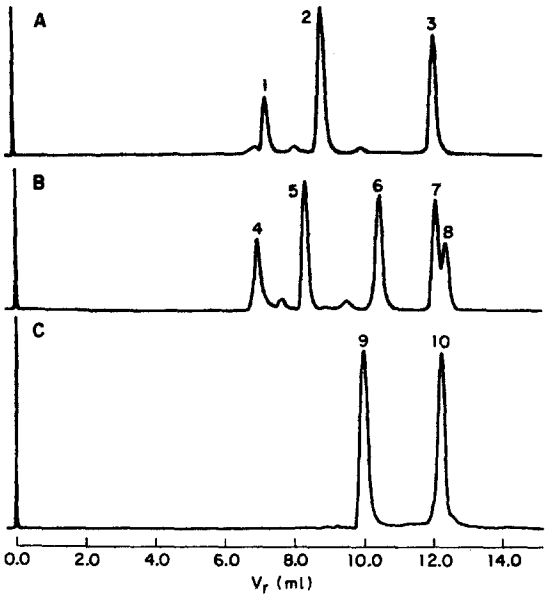

Fig. 8. Separation of proteins on zirconium-stabilized GF-250 DIOL. A, peak identities: (1) ferritin (MW 440 000), (2) ovalbumin (MW 43 500), (3) cytochrome *c* (MW 12 200). B, (4) thyroglobulin (MW 660 000), (5) bovine serum albumin (MW 69 000), (6) myoglobin (MW 17 500), (7) impurity, (8) sodium azide (MW 65). C, (9) β-lactoglobulin (MW 36 000), (10) ribonuclease (MW 13 500). Mobile phase: 0.2 *M* sodium chloride, 0.01 *M* Tris, 0.005% sodium azide pH 7.67. Flow-rate: 1.0 ml min^{-1}. Temperature, ambient. Inlet pressure 550 p.s.i.g. Detector: UV absorbance, 0.64 a.u.f.s. (225 nm).

TABLE III

CHROMATOGRAPHIC PARAMETERS FOR GEL FILTRATION OF PROTEINS

Operating conditions are shown in the legend of Fig. 8.

Sample component	*MW (Daltons)*	*V_r (ml)*	*Plates per column*	*Plates per meter*	*Reduced plate height*	*Skew*
Thyroglobulin	660 000	6.98	6056	24 224	10.3	0.00
Ferritin	440 000	7.28	9902	39 608	6.31	−0.16
Bovine serum albumin	69 000	8.35	12 675	50 700	4.93	0.10
Ovalbumin	43 500	8.92	10 494	41 976	5.96	0.54
β-Lactoglobulin	36 000	9.97	9118	36 472	6.85	0.53
Myoglobin	17 500	10.49	12 244	48 976	5.10	−0.35
Ribonuclease	13 700	12.22	16 309	65 236	3.83	0.01
Cytochrome *c*	12 200	12.15	19 657	78 628	3.18	0.00
Sodium azide	65	12.43	−*	−*	−*	−*

* Impurities in samples obviated precise measurement of parameters near V_m.

affect the separation of proteins on gel columns: ion-exchange interactions and hydrophobic interactions. While these latter mechanisms can be minimized by mobile phase choice, it is difficult to eliminate them totally. Therefore, log MW *versus* elution volume (V_r) plots are not expected to be exactly linear. Fig. 8 shows the separations of a number of commercially available proteins. Chromatographic parameters are tabulated in Table III. The calibration plot of log MW *versus* V_r, derived from these chromatograms, is shown in Fig. 9 and demonstrates the quasi-linear relationship typical of gel chromatography separations of proteins.

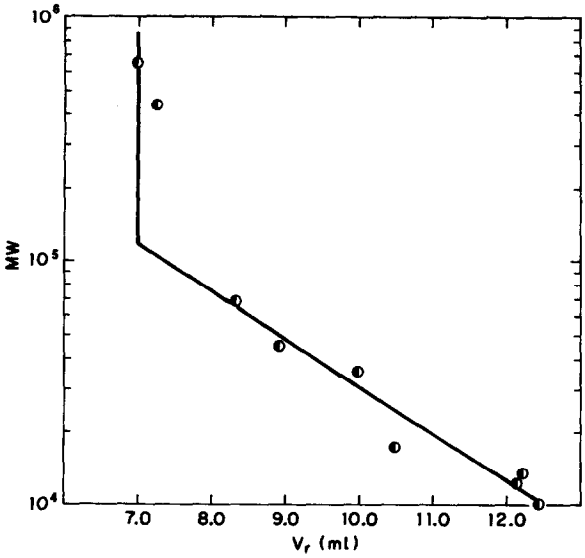

Fig. 9. Gel chromatography calibration plot. Log MW *versus* V_r. Data used to construct the plot were taken from Table IV. For conditions see legend to Fig. 8.

TABLE IV

ELUTION VOLUME (V_r) AS A FUNCTION OF MOBILE PHASE pH AND IONIC STRENGTH (M)

Sample	*pH 8.25 0.2 M*	*pH 7.67*				*pH 7.0 0.2 M*	*pH 6.0 0.2 M*
		0.4 M	*0.2 M*	*0.05 M*	*0.025 M*		
Thyroglobulin	6.95	7.02	6.98	7.03	6.94	6.97	6.98
Ferritin	7.27	7.42	7.28	7.23	7.01	7.46	7.35
Bovine serum albumin	8.24	8.58	8.35	7.66	7.28	8.43	8.56
Ovalbumin	8.85	9.19	8.92	8.06	7.44	9.16	9.12
β-Lactoglobulin	9.79	10.17	9.97	8.83	8.04	9.93	10.39
Myoglobin	10.23	10.64	10.49	10.09	9.57	10.82	11.73
Ribonuclease	11.99	12.28	12.22	11.59	10.99	12.33	12.42
Cytochrome *c*	12.02	12.31	12.15	11.76	10.94	12.62	12.46
Sodium azide	12.27	12.62	12.43	11.63	10.99	12.39	12.53

In another series of experiments, the chromatographic behavior of this packing was examined as a function of pH and ionic strength. Some of these results are shown in Table IV. Fig. 10 shows the effect of varying the ionic strength of the mobile phase at constant pH on the retention volume of several proteins. The figure indicates that some of these substances are influenced by ionic strength to a different degree than others. β-Lactoglobulin is the most affected protein under these conditions, while the retention volume of thyroglobulin remains the same. Thyroglobulin (MW 660 000) is excluded, as expected, but ferritin (MW 440 000) is slightly retained due to secondary effects which are influenced by mobile phase ionic strength.

The same general observations were made when the ionic strength was held constant (0.2 M) and the pH was varied from 6.0 to 8.25. These results (not shown) produced a plot similar to Fig. 10, except that myoglobin was influenced the most.

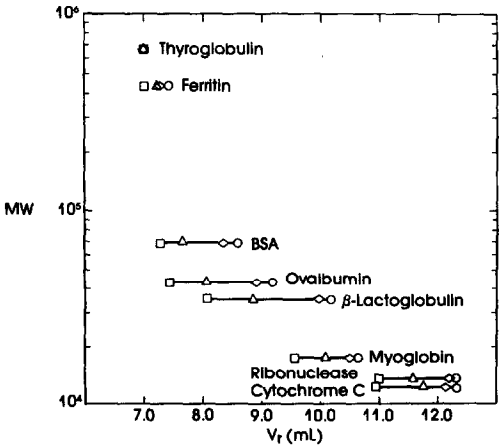

Fig. 10. Effect of mobile-phase ionic strength on elution volume of proteins at pH 7.67: 0.025 M sodium chloride (□), 0.05 M sodium chloride (△), 0.20 M sodium chloride (◇), 0.40 M sodium chloride (○).

Engelhardt and Mathes[8], as well as Roumeliotis and Unger[4,5] have found no correlation between V_r, pH, ionic strength, and physical properties of proteins, such as pI. Our findings are consistent with previous observations in this respect.

CONCLUSIONS

A new approach for the stabilization of bonded-phase silica packings for gel chromatography of proteins has been examined. The silica surface can be modified with a zirconium salt at low pH in aqueous media. The zirconium oxychloride ions are preferentially adsorbed on geminal silanol sites. After thermal treatment in air at 800°C, or higher, the metal oxide is fused to the surface. The calcined silica is then bonded with a monolayer of a hydrophilic organo-silane at pH 8.8 with a reaction temperature of 90°C. The resultant product has a surface coverage of about 1 μmole/m^2 of metal oxide and approximately 4 μmoles/m^2 of DIOL-bonded phase.

Stability experiments were conducted to quantitatively monitor the potential degradation of this packing at elevated pH. A control packing material, without zirconium treatment, was included as was a commercially available column, the TSK G3000SW. We have demonstrated that the described use of zirconium oxide significantly enhances the useful operating lifetime of the column when compared to conventional columns obtained in the marketplace. The zirconium oxide apparently blocks sites on the surface which would normally be attacked by hydroxide ions.

The chromatographic performance of this new packing material has been examined under a wide range of mobile phase ionic strength and pH conditions.

The data presented here indicate that the zirconium-treated surface substantially reduces the restraints in operating conditions that have been common in gel chromatography of proteins. This effect is most obvious in allowing extended operations at pH levels equal to or greater than 8 without degrading the column performance.

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