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HYDROLYZED MACROPOROUS GLYCIDYL METHACRYLATE–ETHYLENE DIMETHACRYLATE COPOLYMER WITH NARROW PORE SIZE DISTRIBUTION

A NOVEL PACKING FOR SIZE-EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

By means of chromatographic methods and mathematical treatment, commercial inorganic and polymeric packings were compared with macroporous glycidyl methacrylate–ethylene dimethacrylate copolymers prepared by the usual polymerization techniques. It was found that the pore size polydispersity of the usual polymeric sorbents is several times higher than that of the inorganic sorbents. For this reason, they are better suited for the separation of mixtures of macromolecules with freely mobile chains than of globular proteins. This was demonstrated by the separation of eleven polystyrene standards and benzene, which was identical with that which can be accomplished only by using a mixed sorbent consisting of several types of silica. Purposeful interferences with the polymerization mixture, *i.e.*, a change in the porogenic mixture, allowed synthesis of polymeric beads in which the pore size distribution was almost identical with that of inorganic packings.

INTRODUCTION

Macroporous polymeric sorbents for size-exclusion (high-performance) liquid chromatography (SEC) are relatively often used in the separation, purification and analysis of proteins, polysaccharides, polynucleotides and other natural polymers, and

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also of many types of synthetic polymers. Some of the best known commercial products, are Separon HEMA (Tessek, Prague, Czechoslovakia)[1], Bio-Gel TSK and Fractogel TSK, both products of TOSOH Japan (TSK GEL PW) distributed by Bio-Rad (Richmond, CA, U.S.A.)[2] and Merck (Darmstadt, F.R.G.)[3] and based on hydrophilic polymers of the methacrylate or polyether type. Compared with inorganic sorbents, especially porous glass and silica, their advantage consists in an high chemical stability in basic media and in a comparatively large pore volume.

With regard to their application in SEC, decisive roles are played by the pore geometry, specific surface area, the average pore size and the pore distribution. These factors also govern the efficiency and selectivity in the separation of macromolecules[4,5]. The structures of inorganic (except glass) and polymeric matrices do not differ in morphology to any important extent, even though their origins are different, but there are considerable differences between the chromatographic properties of the two types of sorbents. The narrowest pore size distribution can undoubtedly be observed in porous glass, but such materials are not available in the particle sizes needed for HPLC. Silica-based sorbents may also have very similar pore properties[6]. Polymeric macroporous sorbents are manufactured by suspension radical polymerization[7]. In the last two cases the porous structure consists of mutually linked submicroscopic globules, which in the case of silica are completely rigid and non-porous.

The arrangement of globules in an individual polymer particle (bead)[8], their sizes and porosities are the main factors which affect the chromatographic properties of a polymeric sorbent. Decisive roles are played by the polymerization conditions and by the composition of the polymerization mixture. Under the usual conditions, the pore size distribution in macroporous polymers is much broader than that observed with inorganic sorbents. For this reason, synthetic polymeric sorbents can on the whole be successfully employed in the separation of macromolecules having a broad molecular weight distribution[2], but they are not suited to the SEC of globular proteins with a narrow size range of macromolecules. This conclusion is reached by comparing the selectivity of separation on polymeric sorbents (TSK GEL PW) and silica-based sorbents with polymeric coatings (TSK GEL SW)[10].

The porous structure of the sorbents affects the separation not only in SEC, but also when modified sorbents are used in other types of HPLC, *e.g.*, hydrophobic interaction, reversed phase and ion exchange[11]. The presence of pores with diameters below about 10 nm markedly lowers the extent of surface modification, and this is negatively reflected in the chromatographic separation.

Thus, there is a need to obtain a polymeric sorbent for the effective SEC separation of rigid globules of proteins, having porous properties close to those of the narrow pore size of inorganic sorbents, but with the additional advantage of being stable also in basic media. A possible route towards this is a variation of the solvent ratio in the porogenic mixture[12,13], and a means of characterizing the required effect is mathematical treatment of the results of chromatographic measurements, *i.e.*, a procedure which is basically identical with the intended application; the results obtained can be applied directly in chromatography.

EXPERIMENTAL

Macroporous copolymers, glycidyl methacrylate–ethylene dimethacrylate (GMA–EDMA), were prepared by the suspension radical polymerization of the two monomers in the presence of a mixture of cyclohexanol and dodecanol as porogenic solvents, and of azobisisobutyronitrile as an initiator. Porogens cause phase separation during cross-linking polymerization, leaving pores in the structure of the final beads. After polymerization had been completed, they were removed from the polymer by washing. A 1% (w/w) solution of poly(vinyl alcohol) in water, which ensures particles with sizes suitable for application in HPLC, was the dispersing phase. The synthesis has been described in detail elsewhere[7,12,13]. The epoxy groups of the macroporous polymer are relatively hydrophobic, but they are reactive, and their presence may lead to irreversible covalent binding of the substances with the sorbent during the chromatographic separation. This is why, prior to packing of the column, the epoxy groups were hydrolyzed in dilute sulphuric acid[9], which gives rise to two vicinal hydroxy groups, increases the hydrophilicity and prevents any chemical reactions that might take place. The extent of hydrolysis was monitored by IR spectroscopy until all epoxides had disappeared from the sorbent. Fractions of the particles were obtained by using an air classifier Multi-Plex Labor Zick-Zack Sichter A 100 MZR (Alpine, Augsburg, F.R.G.), and their widths were determined by means of a Coulter Counter TA II apparatus (Coulter Electronics, Luton, U.K.).

Chromatographic experiments were performed in a TriRotar SR 2 liquid chromatograph (Jasco, Japan) using a RIDK 101 refractometric detector (Laboratory Instruments, Prague, Czechoslovakia) and a spectrophotometric detector UVIDEC IV (Jasco). The columns, 250 mm × 4.6 mm I.D., were packed with sorbents from a slurry in chloroform or acetone under a pressure of 20 MPa. Polystyrene standards (Waters Assoc., Milford, MA, U.S.A.) in tetrahydrofuran (THF) or chloroform (concentration 0.05%, w/w) and dextran standards (Pharmacia, Uppsala, Sweden) in an aqueous solution, concentration 0.5% (w/w) were used for testing.

The porometric characteristics of the sorbents tested were calculated using an approach described in detail in the literature[14,15]. It is based on the measurement of the distribution coefficient, K_d, which is a measure of changes in the free energy of a macromolecule accompanying its penetration into the pores and depends on the coil radius, r, of the polymer molecule of the standard in the given solvent which is related to the molecular weight. The calculation minimizes the sum of the squares of deviations of experimental points from the theoretical curve by variation of the parameters of the model distribution function while applying the experimental dependence of K_d on r/R, where R is the pore radius. To achieve a better approximation to the real system, a method derived for sorbents with non-uniform porosity was used[15]. In this way, the specific surface area, S_g, and the specific pore volume, V_p, were obtained, along with the average pore radius, D_S, calculated from the specific surface area, and also calculated using the pore volume, D_V, and the normalized pore volume V_p/V_t, where V_t is the total sorbent volume in the column.

Using these values, it is possible to calculate the specific pore volume related to the known sorbent density, $V_\rho = V_p/(V_{col} - V_t)$ where V_{col} is the column volume, or the specific pore volume related to the weight of the sorbent $V_w = V_p/w$, and hence also the density of the polymeric sorbent, ρ. Another characteristic quantity is the

specific surface area, $S_{g\rho}$ and S_{gw}, which is obtained as the product of the specific volume, V_ρ or V_w, and of the slope of the initial part of the curve representing the dependence of K_d on r/R mentioned above[15].

The real mixture of polystyrene standards was separated in a microcolumn chromatograph KHZH 1309 (Science and Technology Corporation, Academy of Sciences of the U.S.S.R., Leningrad, U.S.S.R.) provided with a refractometric detector and fluoroplastic columns 350 mm × 0.5 mm I.D., packed either with the sorbent GMA–EDMA (5:95), or with a mixture of porous narrow-dispersion silica gels having a linear calibration graph[16]. The chromatographic analysis took place in 2-butanone, the concentration of the individual components of the mixture being 0.1% (w/w).

RESULTS AND DISCUSSION

Table I collects the data on the composition of the organic phase, used in the preparation of a series of copolymers differing in their cross-linking under otherwise constant reaction conditions. The epoxy groups of these copolymers were hydrolyzed prior to further application.

The procedure chosen for the evaluation of chromatograms of standard compounds (dextrans, polystyrenes) offers the possibility to compare the porometric characteristics of various sorbents used in HPLC, whether inorganic or polymeric. The comparison is however not restricted to the usual characteristics such as the specific surface area, pore volume, pore size distribution, etc., obtained by the B.E.T. method, solvent regain or porosimetry, and mostly in the dry state, *i.e.*, under conditions very far from those prevailing in real applications in various solvents. Table II summarizes the data which characterize the pore properties of a set of commercial inorganic (silica) and polymer sorbents, comparing them with sorbents based on GMA–EDMA. It is clear that there is a considerable difference between the widths of the pore size distributions of inorganic and of polymeric sorbents as characterized by D_V/D_S. Pores

TABLE I

COMPOSITION OF THE ORGANIC PHASE USED FOR SYNTHESIS OF GLYCIDYL METHACRYLATE–ETHYLENE DIMETHACRYLATE SORBENTS

Reaction conditions for polymerization: aqueous phase, 1% (w/w) solution of poly(vinyl alcohol), 60% (v/v), of feed; polymerization temperature 70°C; time 8 h; monomers to porogen mixture ratio 40:60 (v/v).

Sorbent	*Composition of organic phase (%, v/v)*[a]			
	GMA	*EDMA*	*CyOH*	*DoOH*
G-60	60	40	91	9
G-60-WD	60	40	100	0
G-20	20	80	91	9
G-20-WD	20	80	100	0
G-5	5	95	91	9
G-5[b]	5	95	91	9

[a] Abbreviations: GMA = glycidyl methacrylate; EDMA = ethylene dimethacrylate; CyOH = cyclohexanol; DoOH = dodecanol.

[b] Reproducibility test.

smaller than 10 nm and larger than 75 nm are present in polymeric sorbents to a considerable extent. The presence of very small pores as demonstrated below substantially reduces the pore volume accessible to globular proteins (up to 40% of the nominal pore volume), and thus restricts application in the chromatography of proteins. On the contrary, the presence of very large pores, which is in essence due to defects in the steric arrangement of globules in the particle, has been already demonstrated by scanning electron microscopy (SEM)[13]. They are the reason for a lower selectivity in steric exclusion of macromolecules of any type in the molecular weight range 10^2–10^6. The standard sorbents can be still used with advantage in the analysis of mixtures of synthetic polymers containing components having molecular weights above 10^6. The pore size polydispersity of silica-based sorbents is much lower; D_V/D_S lies in the range 1–2, and is thus three to five times lower than that for the usual organic sorbents. It should be stressed, however, that the fraction of pores in unit sorbent volume, V_p/V_t, is not very different for different sorbents, lying in the range 0.5–0.6 in most cases.

In the chromatographic analysis of polymer mixtures possessing a broad molecular weight distribution the high selectivity of silica-based sorbents for proteins is in fact a disadvantage. To make these sorbents suitable for this purpose, chromatographic columns are packed with a mixture of several types of silica with various exclusion limits; their composition must be calculated so as to make the calibration graph for the column linear over the whole range[16]. Fig. 1 shows the calibration dependence of a mixture of silica gels, and also a similar plot for the GMA–EDMA copolymer (G-5). The two plots are very similar. Hence, the sorbent G-5 may be used directly, instead of a laboriously obtained mixture of silica-based sorbents. It can also be seen in Table I that the properties of G-5 and the "linear mixture" are similar. An important advantage of G-5 over silica is the demonstrated stability of the polymer in basic media at usual temperatures[17], whilst their mechanical properties are similar. Fig. 2 illustrates the chromatographic separation of a mixture of eleven polystyrene standards and benzene in a single column packed with the sorbent G-5, which can be regarded as satisfactory.

In the separation of globular proteins, however, the universality for the whole usual range of molecular weights outlined above is not advantageous. Hence we have a challenging task, namely to reduce the pore size distribution to the level of inorganic sorbents while preserving the original pore volume. It has been demonstrated that, in addition to the concentration of the cross-linking agent and temperature, the morphology of macroporous copolymers GMA–EDMA is also affected by the composition of the porogenic mixture[13]. With increasing amount of dodecanol in a mixture with cyclohexanol the specific surface area (B.E.T.) decreases while the pore volume remains the same (solvent regain); bearing in mind the SEM results, it is seen that the presence of dodecanol in the porogenic mixture leads, in particular, to the formation of large pores. The reason is a poorer thermodynamic quality of the porogenic solvent containing dodecanol, resulting in a rapid precipitation of microgels after the onset of polymerization (so-called nuclei which increases to reach the size of globules during subsequent polymerization). Removal of dodecanol from the polymerization mixture will be reflected in a better packing of globules in the macroporous particle and in the elimination of large pores. Two sorbents denoted WD (without dodecanol) were therefore synthesized under identical conditions (with the

TABLE II

CHARACTERISTICS OF THE POROUS STRUCTURE OF INORGANIC AND POLYMERIC SORBENTS FOR SEC[a]

Sorbent	*Producer*	*Material*	D_S *(nm)*	D_V *(nm)*	D_V/D_S	V_p/V_t	V_p *(ml/g)*	S_{gp} *(m^2/g)*	V_w *(ml/g)*	S_{gw} *(m^2/g)*	ρ *(g/ml)*
Lichrospher Si 300	Merck, F.R.G.	Silica	25.0	34.0	1.35	0.60	2.40	180	2.00	165	2.2[b]
Lichrospher Si 500	Merck, F.R.G.	Silica	35.0	49.0	1.40	0.52	1.34	75	–	–	2.2[b]
MPS 250	Gorkii, U.S.S.R.	Silica	16.5	25.5	1.53	0.38	0.60	70	–	–	2.2[b]
Linear mixture L4	Ref. 14	Silica	11.1	51.3	4.58	0.41	0.64	100	–	–	2.2[b]
TSK SW 3000	Toyo Soda, Japan	Silica	13.0	15.0	1.15	0.60	1.75	260	–	–	–
TSK PW 3000	Toyo Soda, Japan	Polymer	7.2	29.2	4.05	0.44	–	–	–	–	–
Separon HEMA BIO 300	TESSEK, Czechoslovakia	Polymer	8.8	36.8	4.15	0.38	–	–	0.63	140	1.3[c]
Separon HEM BIO 1000	TESSEK, Czechoslovakia	Polymer	16.5	84.6	5.10	0.52	–	–	1.34	165	1.4[c]
G-60		Polymer	16.5	85.6	5.19	0.53	–	–	1.55	150	1.45[c]
G-20		Polymer	10.0	42.7	4.25	0.54	–	–	1.50	300	1.44[c]
G-5		Polymer	8.2	41.9	5.09	0.54	–	–	1.55	375	1.46[c]
G-5[d]		Polymer	8.0	42.0	5.25	0.56	–	–	1.58	375	1.45[c]
G-60-WD		Polymer	17.3	41.5	2.40	0.56	–	–	1.17	135	1.45[c]
G-60-WD[d]		Polymer	16.6	49.3	2.95	0.48	–	–	1.0	120	1.50[c]
G-20-WD		Polymer	9.0	20.6	2.30	0.54	–	–	1.12	255	1.43[c]

[a] For abbreviations see text.
[b] Published data.
[c] Calculated data.
[d] Reproducibility test.

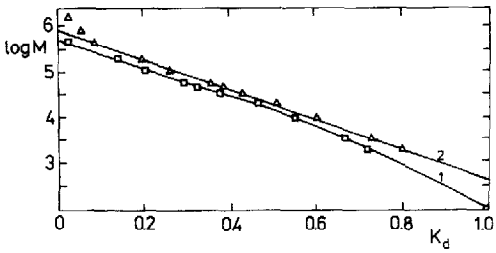

Fig. 1. SEC calibration graphs of the sorbent G-5-(hydrolyzed) (1) and a mixture of silicas (2) for polystyrenes. Conditions: particle size 4–6 μm; metal column 250 mm × 4.6 mm I.D.; eluent THF; elution rate 0.5 ml/min; refractometric detection.

exception of the composition of the porogen). As shown in Table II, the polydispersity of pore size in sorbents of this series does indeed approach that of inorganic sorbents. Fig. 3 demonstrates the pore size distributions of WD sorbents, obtained by mathematical treatment of chromatograms of standards[14,15] and compares them with the distribution of sorbents prepared in the presence of dodecanol. It is clear that the fraction of large pores does indeed considerably decrease but the content of small pores decreases, too. This then leads to a shift of the maximum of the differential distribution curve to somewhat higher values, but the distribution becomes much narrower. The largest fractions in the sorbents G-60-WD and G-20-WD are, respectively, those of pores with sizes 25–27 and 12–16 nm. Pores having radii above 50 nm are hardly present at all in any of the sorbents of the WD series.

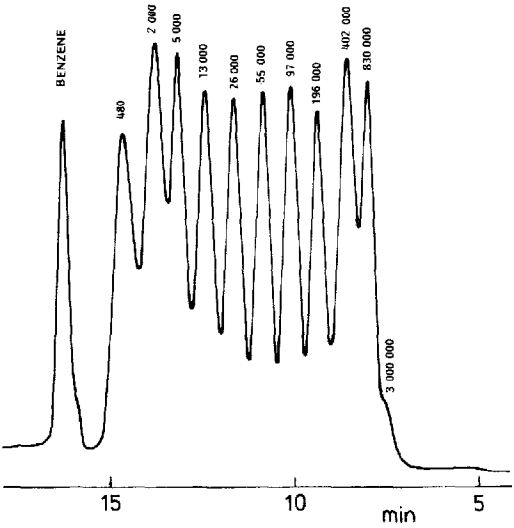

Fig. 2. Size-exclusion HPLC of a mixture of polystyrene standards and benzene. Conditions: sorbent G-5 (hydrolyzed), 4–6 μm; eluent 2-butanone; flow-rate 3 μl/min; PTFE column 350 mm × 0.5 mm I.D.; analysis time 17 min.

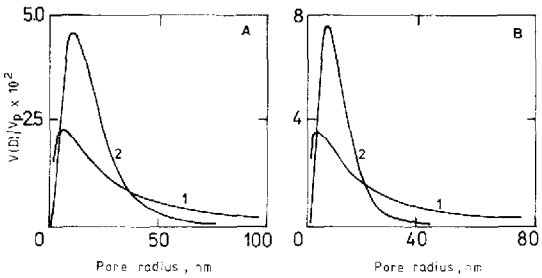

Fig. 3. Effect of the porogenic mixture composition on the pore size distribution of sorbents based on copolymers GMA–EDMA, calculated from chromatographic data. Sorbents: (A) 1 = G-60, 2 = G-60-WD; (B) 1 = G-20, 2 = G-20-WD.

CONCLUSIONS

It has been demonstrated that polymeric sorbents possessing advantageous properties for SEC can be synthesized. A sorbent based on GMA–EDMA (G-5) can replace a mixture of silicas for the separation of polymers with a broad molecular weight distribution. By modifying the composition of the polymerization mixture in the copolymerization of GMA and EDMA, sorbents can be obtained the porous characteristics of which approach those of silica-based commercial sorbents and which have no analogy in commercial polymeric sorbents.

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REFERENCES

1 J. Čoupek, in T. C. Gribnau, J. Visser and R. J. F. Nivard (Editors), *Affinity Chromatography and Related Techniques*, Elsevier, Amsterdam, 1982, p. 165.
2 *Chromatography, Electrophoresis, Immunochemistry, Molecular Biology, HPLC and Liquid Handling*, Catalog N, Bio-Rad Laboratories, Richmond, CA, 1988, p. 52.
3 M. Gurkin and V. Patel, *Am. Lab.*, January (1982) 1.
4 W. W. Yan, J. J. Kirkland, D. D. Bly and H. J. Stoklasa, *J. Chromatogr.*, 125 (1976) 219.
5 J. J. Kirkland, *J. Chromatogr.*, 125 (1976) 231.
6 K. Unger, J. Schick-Kalb and K.-F. Krebs, *J. Chromatogr.*, 83 (1973) 5.
7 F. Švec, J. Hradil, J. Čoupek and J. Kálal, *Angew. Makromol. Chem.*, 48 (1975) 135.
8 Z. Pelzbauer, J. Lukáš, F. Švec and J. Kálal, *J. Chromatogr.*, 171 (1979) 10.
9 T. B. Tennikova, D. Horák, F. Švec, J. Kolář, J. Čoupek, S. A. Trushin, V. G. Maltzev and B. G. Belenkii, *J. Chromatogr.*, 435 (1988) 357.
10 Y. Kato, K. Komiya, H. Sasaki and T. Hashimoto, *J. Chromatogr.*, 193 (1980) 311.
11 T. B. Tennikova, L. V. Vinogradova, V. N. Zgonnik and B. G. Belenkii, *Izv. Akad. Nauk SSSR, Ser. Khim. Nauk*, (1987) 352.
12 D. Horák, F. Švec, M. Ilavský, M. Bleha, J. Baldrian and J. Kálal, *Angew. Makromol. Chem.*, 95 (1981) 117.
13 D. Horák, Z. Pelzbauer, M. Bleha, M. Ilavský, F. Švec and J. Kálal, *J. Appl. Polym. Sci.*, 26 (1981) 411.
14 A. A. Gorbunov, L. Ya. Solovyeva and V. A. Pasechnik, *Vysokomol. Soedin., Ser. A*, 26 (1984) 967.
15 A. A. Gorbunov, L. Ya. Solovyeva and V. A. Pasechnik, *J. Chromatogr.*, 448 (1988) 307.
16 V. V. Nesterov, S. P. Zhdanov, B. I. Venzel, L. Z. Vilenchik, O. I. Kurenbin, T. P. Zhmakina and B. G. Belenkii, *Vysokomol. Soedin., Ser. B*, 26 (1984) 163.
17 J. Hradil and F. Švec, *Reactive Polymers*, 3 (1985) 91.