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SYNTHESIS AND EVALUATION OF CROSS-LINKED POLY(ACRYLOYLMORPHOLINE) SUPPORTS FOR THIN-LAYER GEL PERMEATION CHROMATOGRAPHY

R. EPTON, S. R. HOLDING and J. V. McLAREN
Department of Physical Sciences, The Polytechnic, Wolverhampton, WV1 1LY (Great Britain)
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SUMMARY

A range of poly(acryloylmorpholine) gel networks have been synthesized by aqueous suspension polymerization of acryloylmorpholine and N,N'-methylenediacrylamide in differing molar ratios and at different monomer dilutions. Thin-layer techniques and dyed protein standards were employed to evaluate the networks directly as matrices for aqueous gel permeation chromatography. Increase in the Wheaton and Baumann distribution coefficient, K_d, with buffer content of the gel networks was found to hold only when the ratio of acryloylmorpholine to cross-linker was kept constant or was relatively high. Maximum molecular weight exclusion limit ($> 2 \times 10^6$ for dyed dextran) was observed at high cross-linker concentration.

INTRODUCTION

We have described recently the synthesis of two cross-linked poly(acryloylmorpholine) networks (Enzacryl® Gel K1 and Enzacryl Gel K2) and their evaluation as matrices for gel permeation chromatography (GPC)[1,2]. These networks are applicable in water and a number of organic solvents (*e.g.*, chloroform and tetrahydrofuran) and may be termed "universal" GPC supports. In this respect they are comparable to cross-linked poly(2-hydroxyethyl methacrylate) (Spheron)[3] and the hydroxypropyl derivative of cross-linked dextran (Sephadex LH-20)[4]. The cross-linked poly(acryloylmorpholine) matrix is, however, non-hydroxylic.

The respective molecular weight exclusion limits of Enzacryl Gel K1 and Enzacryl Gel K2 for poly(ethylene glycols) in water are 4,000 and 20,000. For other solvents and solutes the exclusion limits are of similar magnitude. It would be of interest if poly(acryloylmorpholine) packings of much higher exclusion limit could be synthesized.

The two polymerization variables which will most affect the molecular weight fractionation range of a given poly(acryloylmorpholine) network are the molar ratio of acryloylmorpholine to the cross-linker, N,N'-methylenediacrylamide, and the overall aqueous dilution of monomers. In order to study the effects of varying these parameters, we have synthesized a range of aqueous poly(acryloylmorpholine) net-

works and evaluated them directly by thin-layer GPC using dyed proteins as standards. The thin-layer technique enabled us both to expedite our programme and to investigate gels too soft for column use.

EXPERIMENTAL

Materials

Acryloylmorpholine, N,N'-methylenediacrylamide, the surfactants Span 85 (sorbitan trioleate) and Tween 85 [polyoxyethylene(20)sorbitan trioleate], and the scintillators POPOP (*p*-bis[2-(5-phenyloxazolyl)]-benzene) and PPO (2,5-diphenyloxazole) were obtained from Koch-Light Labs. (Colnbrook, Great Britain). Scintillation-grade toluene and Triton X-100 (isooctylphenoxyethanol) were purchased from Nuclear Enterprises (Redhill, Great Britain). Liquid paraffin ($\varrho^{20} = 0.85$ g cm^{-3}; $\eta^{20} = 3.5 - 4.0$ N sec m^{-2}) was supplied by Hopkin and Williams (Romford, Great Britain).

The dyed protein standards, used for characterization of the gels, were prepared as described previously[5].

Synthesis of gel networks

A number of gel networks were prepared. The mole ratio of acryloylmorpholine to N,N'-methylenediacrylamide together with the aqueous dilution of monomers in each polymerization mixture and the nomenclature used to describe the gels produced are summarized in Table I.

TABLE I

COMPOSITION OF POLYMERIZATION MIXTURE AND FINAL BUFFER CONTENT OF CORRESPONDING GEL NETWORKS

Gel nomenclature in parentheses.

Aqueous dilution of acryloylmorpholine (ml·g^{-1})*	*GPC buffer content, V_h, of polymer network (ml·g^{-1})*				
	Mole ratio of acryloylmorpholine to N,N'-methylenediacrylamide				
	5:1	*10:1*	*20:1*	*40:1*	*80:1*
2.8	—	2.9 (K2)	—	—	—
5.7	—	5.1 (K3)	—	—	—
11.4	9.7 (J4)	12.1 (K4)	12.4 (L4)	17.4 (M4)	20.6 (N4)
14.9	—	14.9 (K5)	—	—	—

* Amount of water includes that of the aqueous potassium persulphate solution used to initiate polymerization.

Bead polymerization was carried out in a 2-l glass polymerization flask equipped with a large crescent-shaped stirrer paddle which just swept the wall of the flask. Liquid paraffin (1 l) containing 4% (v/v) of surfactant was placed in the latter and deoxygenated by bubbling nitrogen vigorously for 3 h with stirring. The aqueous solution of monomers and an initiator solution, consisting of potassium persulphate (2% w/v) in water, were deoxygenated similarly. Then a calculated amount of initiator solution was mixed well with the monomer solution and the resulting aqueous phase

(400 ml) added to the bulk phase in the polymerization flask. A short period of vigorous stirring was sufficient to disperse the aqueous phase into droplets of appropriate size. Then the stirring rate was reduced. A nitrogen atmosphere was maintained in the flask.

Usually, the surfactant Span 85, HLB 1.8 (ref. 6), was effective in preventing bead agglomeration but in the case of gel network J4 a mixture, HLB 3.0, of Span 85 and Tween 85 (20:3) was required. In most bead polymerizations the aqueous phase was dispersed such that maximum droplet diameter was just less than 50 μm. However, in the case of gel networks M4 and N4 droplet diameter was kept below 40 and 35 μm, respectively, to compensate for swelling which occurred on equilibrating the networks with the GPC buffer.

To obtain reproducible polymerization times it was necessary to predetermine the amount of initiator for each bead polymerization in a preliminary, small-scale, block polymerization by means of a reciprocating piston gel timer (Tecam, Cambridge, Great Britain). An initiator concentration which would stop the gel timer in 10–20 min was found convenient. At this concentration a temperature maximum was observed between 30 min and 1 h in the corresponding bead polymerization.

Polymerization was allowed to go to completion overnight after which the poly(acryloylmorpholine) gel beads were washed free of paraffin and surfactants with light petroleum (b.p. 40–60°). The beads were then rinsed with acetone and equilibrated with the GPC buffer. This consisted of a solution of 0.5 *M* NaCl in 0.05 *M* aqueous Na_2HPO_4 (pH 8.6).

Buffer content of gel networks

To determine the amount of buffer contributing to each xerogel, a portion of the equilibrated network was transferred to a Hirsch funnel and the buffer external to the beads drawn off. A sample of the xerogel network was transferred by means of a microspatula and placed on a preweighed microscope slide. The slide was weighed and the weight of the sample of xerogel network (polymer plus buffer component), W_1, calculated. After drying the slide to constant weight it was reweighed and the combined weight, W_2, of polymer and buffer salts which had contributed to the xerogel networks recorded. The amount of water in the sample was thus $W_1 - W_2$.

It was established that an aliquot of the GPC buffer solution containing 1 g of water also contained 0.037 g of buffer salts. The buffer content, V_b, per g of polymer in each xerogel network was calculated from the relationship

$$V_b = \frac{W_1 - W_2}{W_2 - 0.037\,(W_1 - W_2)}$$

Preparation and development of thin-layer plates

Suspensions of the gel beads in the GPC buffer were centrifuged to effect settling and the supernatants discarded. Each slurry so obtained was spread onto a thin-layer plate of 20 × 20 cm to a depth of 0.6 mm. These were then transferred to a thin-layer apparatus (Pharmacia, London, Great Britain), connected to the buffer reservoirs by strips of Whatman No. 3 filter paper and left to equilibrate overnight while running at a 5° slope.

Solutions of the chromatographic solutes (dyed proteins, tritiated water, dyed

Dextran 2000 and dyed cells of *E. coli*) in the GPC buffer were injected into the gel on a level plate. Sample volumes injected did not exceed 5.0 μl and maximum solute concentration was 1 %. Spots of thyroglobulin were placed next to each of the other standards in order that any uneven flow could be observed readily. The plates were developed at a 15–25° slope for from 6–8 h.

Determination of Wheaton and Baumann[7] distribution coefficients

For each solute retarded on thin-layer GPC the Wheaton and Baumann absolute distribution coefficient, K_d, was calculated by means of the relationship[5]

$$K_d = \frac{d_s\,(d_0 - d_c)}{d_c\,(d_0 - d_s)}$$

where d_0 is the zone migration distance of a totally excluded solute, usually dyed Dextran 2000 or dyed *E. coli*, d_s is the migration distance of the pure mobile phase and d_c the migration distance of the given solute. It was assumed that d_s corresponded to the migration distance of tritiated water. The results are presented in Table II.

Detection of chromatographic standards

A permanent record of the position of the dyed chromatographic standards on each developed thin-layer plate was obtained by bringing it into contact with a sheet of Whatman No. 1 filter paper. Most of the solvent together with the dissolved solutes was transferred to the paper, which was then dried. For convenience, the zone migration distance of each dyed standard was measured from this record.

To detect tritiated water on a given plate and so estimate the migration distance of the GPC solvent, bands of gel 0.2 cm wide were scraped from the plate at 0.25-cm intervals at right angles to the direction of flow. Each gel sample obtained was

TABLE II

K_d VALUES OF DYED STANDARDS ON POLY(ACRYLOYLMORPHOLINE) GELS

Absence of data implies faint or diffuse spots.

Dyed standard	*Molecular weight*	*Gel*								
		K2	*K3*	*K4*	*K5*	*J4*	*K4*	*L4*	*M4*	*N4*
Ribonuclease A	13,600	0.11	0.37	0.26	0.75	0.57	0.26	0.64	0.79	0.98
	27,200*	0.03	0.18	0.17	—	0.33	0.17	0.34	0.50	0.61
	40,800**	—	—	—	—	—	—	0.21	0.33	—
Ovalbumin	45,000	0.04	0.12	0.17	0.60	0.41	0.17	0.22	0.34	0.35
	90,000*	—	0.05	0.11	0.35	0.32	0.11	0.05	0.11	0.14
Albumin	67,000	0.01	0.06	0.12	0.44	0.37	0.12	0.08	0.06	0.20
	134,000*	—	0.01	0.06	0.24	0.27	0.06	0.05	0.04	0.08
	201,000**	—	—	0.05	0.16	0.23	0.05	0.02	—	0.02
Fibrinogen	335,000	—	—	0.03	0.14	0.21	0.03	—	0.02	0.00
Thyroglobulin	670,000	0.00	0.00	0.00	0.08	0.11	0.00	0.00	0.00	0.00
Dextran 2000	2,000,000	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00
E. coli	—	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

* Dimer.
** Trimer.

equilibrated with 2 ml of water, centrifuged, and a 1-ml aliquot of the supernatant diluted with 10 ml of scintillation cocktail. The diluted samples were assayed for tritium content by means of a Phillips liquid scintillation counter. The scintillation cocktail consisted of 0.4% PPO and 0.02% POPOP in solution in a mixture (2:1) of toluene and Triton X-100. Gel calibration with tritiated water and a totally excluded solute was carried out on separate thin-layer plates of 5 × 20 cm.

Column GPC

A single-column experiment was performed to confirm that the elution behaviour of the dyed standards was similar to that of the corresponding native proteins. A glass GPC column (Jobling Laboratory Division, Stone, Great Britain) of 1 × 100 cm was packed in the GPC buffer with a batch of gel network K4 specially synthesized to contain beads of diameter 75–150 μm. The native proteins thyroglobulin, albumin, ovalbumin and ribonuclease A and their purified dyed equivalents were run sequentially through the column. The column effluent was monitored spectrophotometrically at 280 and 475 nm to detect the native proteins and dyed equivalents, respectively. The elution volumes are recorded in Table III.

TABLE III

ELUTION VOLUMES OF NATIVE AND DYED PROTEINS

Each dyed protein solution was carefully purified to remove dimers, trimers and excess dye.

Protein	*Elution volume before dyeing (ml)*	*Elution volume after dyeing (ml)*
Thyroglobulin	32.22	31.85
Albumin	34.16	34.20
Ovalbumin	37.08	35.91
Ribonuclease A	47.72	47.96

RESULTS AND DISCUSSION

All the copoly(acryloylmorpholine) networks synthesized were obtained from the polymerization mixtures as discrete beads with the exception of gel network K5. Some slight bead agglomeration occurred with this gel and as a result the corresponding thin-layer plate developed relatively slowly. This led to the choice of either short migration distance or, if development was carried out over an extended period, diffuse spots. Excellent flow-rates were obtained with the other gel networks.

The degree of swelling of the gel networks in the GPC buffer (Table I) was rather less than in pure water. This was apparent especially with the more expanded networks M4 and N4. Nevertheless, these same networks imbibed much more GPC buffer on equilibration than the volume of water used in their preparation.

Wheaton and Baumann distribution coefficients (Table II) for the various dyed protein standards were calculated for each gel. In the case of albumin, ovalbumin and ribonuclease A the presence of dimers and trimers was invaluable since these behaved effectively as additional standards of known molecular weight. However, the low concentrations of these species sometimes made them difficult to observe. To a lesser

extent this was true of fibrinogen and *E. coli*, neither of which could be dyed so intensely as the typical globular proteins. Where, owing to faint or diffuse spots, a particular migration distance was difficult to measure accurately, it has been omitted from Table II.

It is important to note that the dyeing procedure does not effect significantly the GPC behaviour of the various protein standards. This was confirmed by accurate measurement of the GPC elution volumes (Table III) of a selection of dyed and native proteins on a column packed with a specially prepared batch of gel network K4. Since the elution volume of each protein and its dyed equivalent are within experimental error, the same it is clear that they must have similar hydrodynamic volumes. Major alteration of the hydrodynamic volume of the proteins might have occurred as a result of electrostatic changes arising on attachment of the negatively charged dye. The fractional increase in protein hydrodynamic volume resulting from the additional molecular volume of the attached dye molecules alone would be too small to produce detectable changes in GPC elution volume.

Logarithm molecular weight *versus* K_d plot for the series of gel networks K2, K3, K4 and K5, synthesized at progressively increasing monomer dilution but at constant mole ratio of acryloylmorpholine to cross-linker, are presented in Fig. 1. It is clear that the increase in GPC buffer content on ascending the series is accompanied by a parallel increase in K_d for included solutes and an overall increase in molecular weight exclusion limit. This is not unexpected. Similar results have been recorded by Sun and Sehon[8] in the case of polyacrylamide gels.

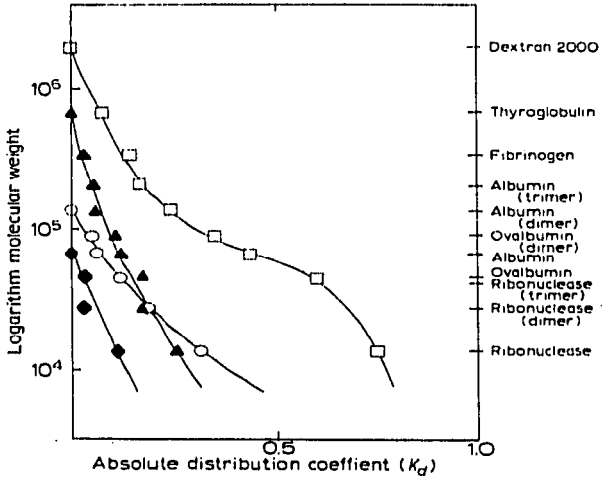

Fig. 1. Logarithm molecular weight *versus* K_d plots for gel networks K2(◆), K3 (○), K4 (▲) and K5 (□) synthesized at progressively increasing dilution of acryloylmorpholine.

Increase in K_d with buffer content of the gel network was found to hold only when the ratio of monomer to cross-linker was kept constant or was relatively high. This is apparent from Fig. 2, in which the logarithm molecular weight *versus* K_d plots for the gel networks J4, K4 ,L4, M4 and N4 are presented. These networks were prepared with progressively increasing mole ratio of acryloylmorpholine to cross-

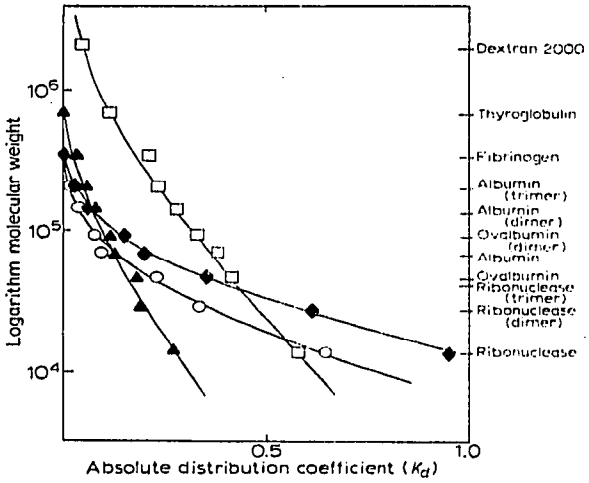

Fig. 2. Logarithm molecular weight *versus* K_d plots for gel networks J4 (□), K4 (▲), L4 (○) and N4 (◆) prepared with progressively increasing mole ratio of acryloylmorpholine to cross-linker. The plot for gel network M4 falls between those for networks L4 and N4 but has been omitted for clarity.

linker but at fixed acryloylmorpholine dilution. If a substantial amount of cross-linker is present, as in the case of network J4, a dramatic increase in molecular weight exclusion limit is observed. The molecular weight exclusion limit actually diminishes across the series J4, K4, L4, even though the equilibrium buffer content of the networks is increasing. Fawcett and Morris[9] have noted similar trends in the exclusion limits of polyacrylamide gels at high cross-linker concentrations.

Two further gels were prepared in an attempt to extend the range of networks studied. One of these, gel network K6, was intended to extend the series synthesized at constant mole ratio of acryloylmorpholine to cross-linker (K2, K3, K4, K5) and was prepared at an aqueous acryloylmorpholine dilution of 22.5 ml g^{-1}. The other, gel network O4, was intended to extend the series synthesized at constant acryloylmorpholine dilution (J4, K4, L4, M4, N4) and had an acryloylmorpholine to cross-linker ratio of 160:1. Gel networks O4 and K6 proved to be extremely soft and lacking in mechanical stability. The thin-layer plate corresponding to network K6 developed so slowly that diffuse, merged spots resulted and consequently meaningful data could not be obtained. The plate made up from network O4 could not be developed at all.

The shape of the logarithm molecular weight *versus* K_d plots is important in the choice of support matrix for a particular GPC application. In preparative GPC, maximum efficiency in separating two components is obtained when they have widely differing K_d values. If the molecular weights of the components are close, the best separations will be obtained using supports which give plots of minimum slope in the molecular weight range of the components. These supports are the lightly cross-linked gel networks (L4, M4, N4). For analytical GPC, gel networks prepared using higher concentration of cross-linker (J4, K4) will be the most useful. These supports cover a wide molecular weight fractionation range. If such materials were used for analytical column GPC then their mechanical stability would permit higher flow-rates than in the case of the softer, lightly cross-linked gels.

The characteristic shape of the logarithm molecular weight *versus* K_d curves is related to gel microstructure[9]. It is probable that at low cross-linker concentrations the supports are homogeneous xerogel networks albeit with a random distribution of pore sizes. With supports incorporating a larger proportion of cross-linker it is probable that chain clustering occurs. Such gel networks will possess a degree of heterogeneity.

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REFERENCES

1 R. Epton, C. Holloway and J. V. McLaren, *J. Chromatogr.*, 90 (1974) 249.
2 R. Epton, C. Holloway and J. V. McLaren, *J. Appl. Polym. Sci.*, 18 (1974) 179.
3 J. Coupek, M. Krivakova and S. Pokorny, *J. Polym. Sci., Part C*, 42 (1975) 185.
4 M. Joustra, B. Soderqvist and L. Fischer, *J. Chromatogr.*, 28 (1967) 21.
5 R. Epton, S. R. Holding and J. V. McLaren, *Polymer*, 15 (1974) 466.
6 W. C. Griffin, *J. Soc. Cosmet. Chem.*, 5 (1954) 249.
7 R. M. Wheaton and W. C. Baumann, *Ann. N.Y. Acad. Sci.*, 57 (1953) 159.
8 K. Sun and A. H. Sehon, *Can. J. Chem.*, 43 (1965) 969.
9 J. S. Fawcett and C. J. O. R. Morris, *Separ. Sci.*, 1 (1966) 9.