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Review

Rigid macroporous copolymers as stationary phases in high-performance liquid chromatography

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ABSTRACT

Rigid macroporous copolymers of styrene and divinylbenzene have been developed which have the high physical/mechanical stability necessary to operate under high-performance liquid chromatographic conditions of pressure and flow-rate. The matrices can be produced in a range of porosities, 100, 300, 1000 and 4000 Å each with the controlled uniform pore geometry and pore size distribution essential for high efficiency separations. The small pore–high surface area macroporous poly(styrene–divinylbenzene) copolymer, PLRP-S 100 Å, can be used in the unmodified form for reversed-phase separations of small molecules or by increasing the pore size, PLRP-S 300 Å or greater, for the analysis of biological macromolecules.

The surface characteristics of the hydrophobic poly(styrene–divinylbenzene) matrix can be altered by derivatization or coating to produce ion exchange functionalities or a hydrophilic surface for gel filtration chromatography. The hydrophilic surface can then be further derivatized to be utilised as a base matrix for affinity chromatography.

CONTENTS

1. INTRODUCTION

The use of rigid macroporous copolymers for high-performance liquid chroma-
tography (HPLC) has its origins in the work carried out by Peterson [1]. In 1954 he
made the observation that proteins could under certain conditions be adsorbed by
diethylaminoethyl (DEAE) derivatized cellulose and then subsequently eluted by
increasing the ionic strength of the eluent. This example of the use of a polymeric anion
exchanger for liquid chromatography was followed by the use of a carboxymethyl
(CM) cellulose derivative for cation exchange chromatography [2] and a cross-linked
polydextran gel which when swollen by aqueous eluents could be used to separate by
size water-soluble biological macromolecules [3]. These materials are still commer-
cially available for low/medium pressure liquid chromatography and are used
extensively for preparative fractionations of biological macromolecules. However,
these microporous polymer gels are lightly cross-linked copolymers in which the
porosity of the particle is determined by the percentage of the cross-linking monomer
used. The lower the percentage of cross-linker the bigger the porosity in the presence of
a good solvent but the softer is the resultant polymer matrix. Therefore these lightly
cross-linked semi-rigid polymer networks are easily compressed and can only be
operated under low/medium pressure. With increasing pressure the polymer network
collapses restricting the flow and reducing the column permeability. Polymers with
a degree of cross-linking of 8% such as the sulphonated poly(styrene–divinylbenzene)
matrices used for free amino acid [4] or oligosaccharide analysis [5] are used with
HPLC equipment but only at elevated temperature and when packed in wide bore
columns. Below this degree of cross-linking they are generally unsuitable for HPLC
separations.

In the 1970s silica became the matrix of choice for HPLC being a rigid
macroporous support able to operate under high pressure/flow and being commer-
cially available in a range of pore and particle sizes. The high flow-rates and favourable
mass transfer characteristics enabled high efficiency separations to be achieved in
a fraction of the time required when using microporous gels. The rigid macroporous
polymers, copolymers with a high degree of cross-linking, were slower to develop than
the inorganic matrices. Moore in 1964 [6] synthesised macroporous poly(styrene–
divinylbenzene) materials with a high percentage of cross-linker, divinylbenzene. He
carried out the polymerization in the presence of a porogen, a compound which is
soluble in the monomers but insoluble in the polymer and produced rigid spherical
particles containing large voids/pores. Porous materials similar to these have been
used for organic phase gel permeation chromatography [7] and subsequently in
microparticulate form for reversed-phase chromatography [8].

The range of rigid macroporous HPLC adsorbents produced by Polymer Labs.
and discussed in this article are based on the technology originally developed by Moore
[6]. The poly(styrene–divinylbenzene) matrices being used in an underivatized form for
reversed-phase chromatography and after derivatization for ion-exchange, gel filtra-
tion and affinity chromatography.

2. PREPARATION AND CHARACTERISATION

Macroporous polymer gels are usually prepared by suspension polymerization
of the monomer or monomers and cross-linking monomer in a water–organic

two-phase reaction system [9]. The reaction mixture consisting of monomers, cross-linking monomer and polymerization initiator in the presence of a suitable porogen are stirred rapidly with water to produce organic phase droplets equivalent to the particle size of the polymer bead required. A suspension stabilizer may be required to prevent the droplets coalescing during polymerization. The polymerization proceeds in the droplets of the organic phase with the growing polymer chains precipitating in the droplet as they reach a critical size. The presence of the cross-linking monomer results in the formation of the rigid three dimensional structure. The porogen voids in the polymer network are the macropores. By suitable choice of porogen the pore size, pore size distribution and pore geometry can be optimized for HPLC separations.

In size-exclusion chromatography (SEC), the separation mechanism is dependent upon the molecular size, hydrodynamic volume, of a solute in solution. Therefore an SEC calibration curve, plot of elution volume *versus* molecular size may be related to the pore size distribution and pore volume of an HPLC adsorbent [10]. Fig. 1 shows the SEC calibration curves for the range of poly(styrene–divinylbenzene) reversed-phase HPLC adsorbents, PLRP-S 100, 300, 1000 and 4000 Å. It can be seen that with the larger pore materials there is a sharp drop off in the calibration curve due to the absence of small pores. This reduces band broadening due to restricted diffusion for larger molecules which can occur when they are chromatographed using small pore materials. For macroporous packings the surface area, total pore volume and mean
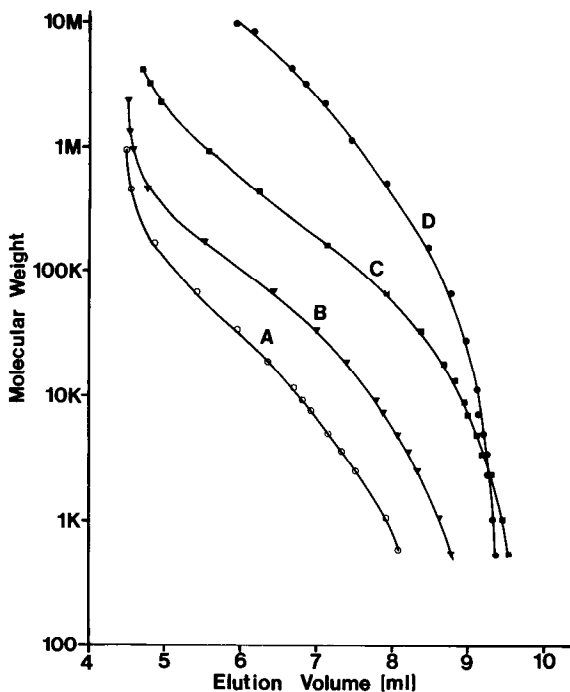

Fig. 1. Size-exclusion chromatography calibration curves for polystyrene standards in tetrahydrofuran at ambient temperature. Column, 300 × 7.5 mm I.D.; flow-rate, 1.0 ml/min; detector, UV operated at 254 nm. (A) 100 Å, 5 μm; (B) 300 Å, 8 μm; (C) 1000 Å, 8 μm; (D) 4000 Å, 10 μm (K = kDa; M = 1000 kDa).

TABLE 1

THE RELATIONSHIP BETWEEN PORE SIZE AND SURFACE AREA, AS DETERMINED BY
BET NITROGEN ADSORPTION ISOTHERMS, FOR THE PLRP-S RANGE OF POLY(STYRENE–
DIVINYLBENZENE) REVERSED-PHASE MATERIALS

Adsorbent	Pore size (Å)	Surface area (m²/g)
PLRP-S	100	414
PLRP-S	300	384
PLRP-S	1000	267
PLRP-S	4000	139

pore diameter can be related. Typical HPLC silica based packing materials with
a mean pore diameter of 300 Å and 100 Å will have surface areas calculated from gas
adsorption isotherms of approximately 200 and 300 m²/g, respectively [11]. However,
from the PLRP-S nitrogen adsorption isotherms, much higher surface areas were
obtained, see Table 1. These high values are due to the biporous structure of the
polymer matrix [12]. The presence of pores of 20 Å in radius or smaller as shown by the
bimodel pore distribution obtained by nitrogen adsorption measurements suggests
that micropores may be present in the network of cross-linked polymer chains which
surround the macropores. These micropores will only be accessible to very small
solutes but would be expected to influence selective binding/adsorption of small rigid
molecules of a certain size or shape. Although it is not possible due to the nature of
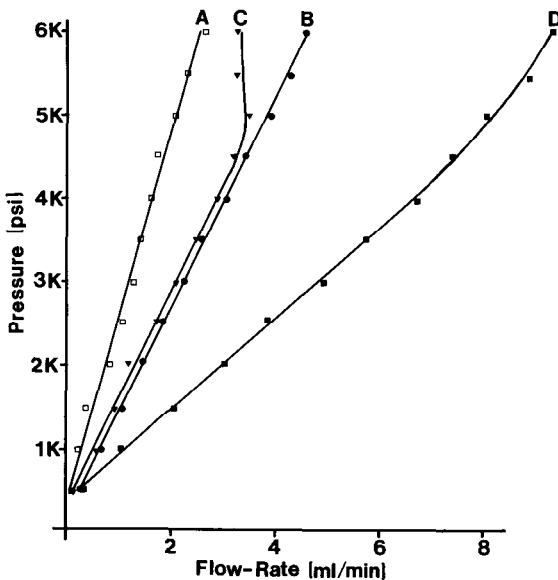formation of the micropores to eliminate them entirely from the macroporous polymer

Fig. 2. Pressure *versus* flow velocity in acetonitrile–water (40:60, w/w) at ambient temperature. Column,
250 × 4.6 mm I.D.; (A) 100 Å, 5 μm; (B) 300 Å, 8 μm; (C) 1000 Å, 8 μm; (D) 4000 Å, 10 μm.

the size and number can be minimized. From the SEC calibration curves for the PLRP-S range of materials there is no plateau evident at low molecular weight so suggesting that the micropores are only accessible to solutes with a hydrodynamic volume less than that of phenylhexane (molecular weight 162) which is the bottom point on the calibration curve.

For HPLC separations the macroporous polymer must be able to operate at high flow-rates when packed in conventional HPLC column hardware of 4.6 mm I.D. This requires both mechanical rigidity and permeability. The stability of the column packing material may be monitored by examining the dependence of column pressure on eluent flow-rate. Fig. 2 shows the plots obtained for the range of PLRP-S adsorbents. A reasonable linear dependency is observed for the four adsorbents up to 4500 p.s.i. suggesting that little particle distortion/compression occurs below this pressure. The difference in the slope of plots is due to the particle size. Above 4500 p.s.i. the two larger pore materials start to compress and the permeability is reduced. Therefore the PLRP-S range of macroporous poly(styrene–divinylbenzene) matrices are all capable of operating under HPLC conditions of pressure and flow-rate.

The influence of pore size and hence surface area on small molecule retention was determined for phenol, aniline, benzene and toluene as a function of eluent composition for the range of PLRP-S pore sizes. Fig. 3 shows the plots of log solute retentivity/capacity factor (k') against mobile phase composition for the four solutes chromatographed using the four different pore sizes. In the case of both phenol and aniline the form of the plots indicates that the available surface area for solute interaction decreased with increasing pore size, *i.e.*, at any given mobile phase composition the retentivity decreased as the pore size of the adsorbent increased. In the case of the benzene and toluene it was observed that the PLRP-S 1000 Å adsorbent appeared to have less available surface area for solute interaction than the PLRP-S 4000 Å material as indicated by the lower solute retentivity. This may be due to differences in the microporous structure causing increased retention of the hydro-
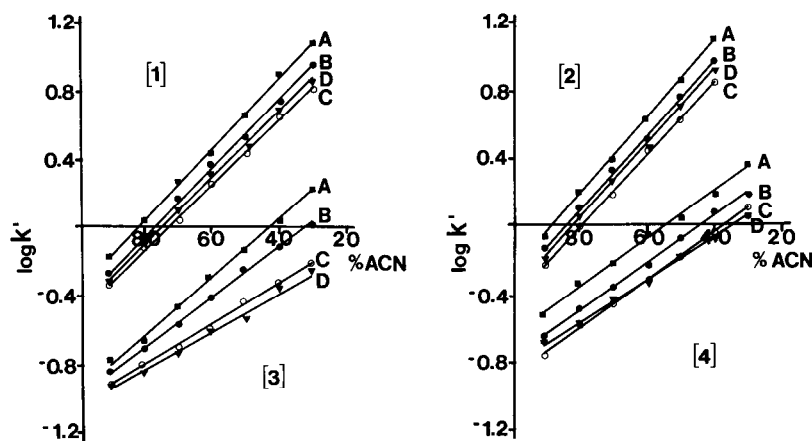

Fig. 3. Dependence of solute capacity factor (k') on eluent composition at ambient temperature. Column, 150 × 4.6 mm I.D.; flow-rate, 1.0 ml/min; detector, UV operated at 254 nm. (A) 100 Å, 5 μm; (B) 300 Å, 8 μm; (C) 1000 Å, 8 μm; (D) 4000 Å, 10 μm. 1 = Benzene; 2 = toluene; 3 = phenol; 4 = aniline.

phobic aromatics. Therefore it would appear that available surface area and hence pore size is an important variable in the control of retentivity with small molecules. Comparing the retentivity of the PLRP-S 100 Å material with small pore octadecyl (ODS) bonded silica with small molecules the hydrophobic poly(styrene–divinyl-benzene) matrix is found to be considerably more retentive [13]. Due to the higher available surface area and "apparent" increased hydrophobicity of the polymer compared to even high carbon load ODS silica materials it is exceptionally useful for pre-concentration work as for example in the determination of phenols [14] and for the reversed-phase analysis of water-soluble hydrophilic species for example vitamin C [15]. The use of exceptionally hydrophobic solutes (where in order to obtain reasonable retention volumes, higher percentages of organic modifier or a stronger organic modifier are used in the eluent, for example in the determination of p-aminobenzoic acid in urine to evaluate exocrine pancreatic function [16]) enables eluents more compatible with sample solubility to be oxidized.

2.1. Column efficiency

A plot of plate height *versus* linear flow velocity produced for the high efficiency, small pore PLRP-S 100 Å 5 μm material in acetonitrile–water (9:1, w/w), Fig. 4, clearly shows that the plate height achieved with the polymer is comparable to that which would be expected for bonded silica packings but that the optimum linear velocity of the mobile phase is smaller. It has been shown that an increase in efficiency/decrease in the plate height value can be achieved by increasing the temperature [8]. However, it should be noted that increasing the temperature also reduces the retentivity. The improved efficiency has been tentatively attributed to an improvement in the restricted diffusion of a solute in the stationary phase and not to the earlier elution of the solute. Experiments have been performed where the temperature is increased but the retention held constant by increasing the aqueous content of the mobile phase (the decrease in liquid viscosity on increasing the temperature will be balanced by an increase in the aqueous content of the eluent, water being more viscous than the organic) therefore it is proposed that increased efficiency with increasing temperature is due to an improvement in the intraparticle mass transfer.
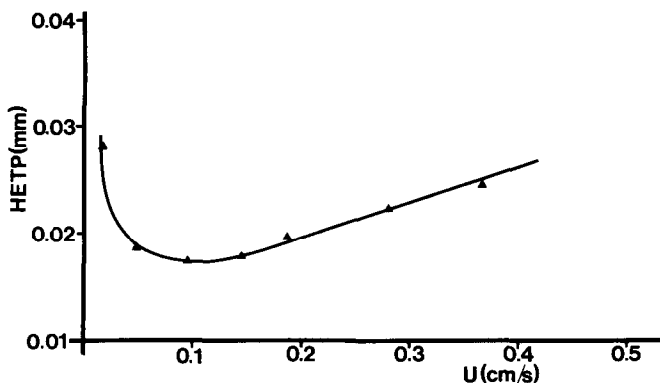

Fig. 4. Dependence of HETP on flow velocity for PLRP-S 100 Å 5 μm at ambient temperature. Column, 200 × 4.6 mm I.D.; detector, UV operated at 254 nm; eluent acetonitrile–water (90:10, w/w); solute, nitrobenzene.

2.2. Eluent compatibility solvent strength

There have been a number of solvent strength studies carried out using macroporous poly(styrene–divinylbenzene) adsorbents [17–21]. Robinson et al. [18] working with XAD materials and benzo[a]pyrene and benz[a]anthracene as non-polar probes reported on eluotropic series with dimethyl sulphoxide (DMSO) being a strong solvent. However, in a subsequent study by Bowers and Pedigo [21] DMSO was shown to be a relatively poor solvent for poly(styrene–divinylbenzene) but a good solvent for benzo[a]pyrenes which influenced the results. In all studies there is agreement that the alcohols are weak solvents with acetonitrile and tetrahydrofuran increasing in strength.

The solvent strength would appear to have a major influence on peak symmetry for non-polar small molecules. Bowers and Pedigo [21] correlated the peak symmetry with the column void volume for a series of eluents of different strength for acetonitrile and tetrahydrofuran, good solvents, the void volume was significantly smaller than for the poor solvents, alcohols and DMSO and the peak shape was significantly improved. This is interpreted as a swelling of the microporous structure of the particle polymer chain network by the good solvent. The asymmetrical peak shapes with poor solvents are more exaggerated as the aromatic nature of the solute increases, increased π–π interactions, however, a significant improvement in peak symmetry can be obtained by the use of a ternary eluent, water, good solvent and poor solvent. If approximately 10% of tetrahydrofuran is added to a poor solvent system then the peak shapes obtained are consistent with those of the good solvent. Fig. 5 shows the separation of a series of alkylphenones with a methanol–water (80:20, v/v) and acetonitrile–water (80:20, v/v) eluent. Increasing the strength of the mobile phase, replacing methanol,
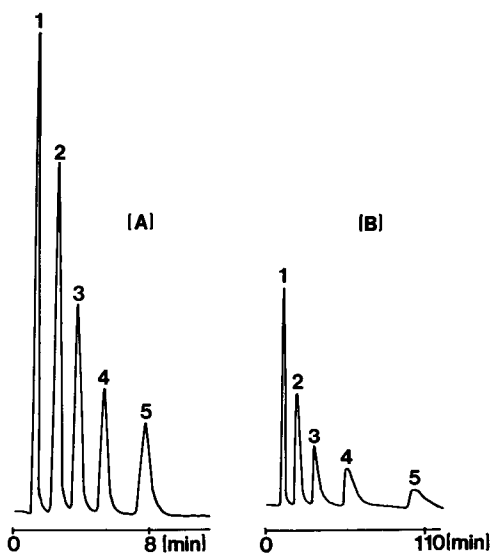

Fig. 5. Separation of alkylphenones using PLRP-S 100 Å 5 μm material at ambient temperature. Column, 150 × 4.6 mm I.D.; flow-rate, 1.0 ml/min; detector, UV operated at 254 nm; eluent (A) acetonitrile–water (80:20, w/w); (B) methanol–water (80:20, w/w). Solutes, 1 = methylphenone; 2 = ethylphenone; 3 = propylphenone; 4 = butylphenone; 5 = pentylphenone.

a poor solvent, with acetonitrile, a good solvent, improves peak symmetry and reduces the analysis time.

In the area of small molecule reversed-phase HPLC the efficiency of separation achieved with the poly(styrene–divinylbenzene) packings does not match that of silica based materials. However, the macroporous polymers do have significant advantages in the area of chemical stability where the silica base matrix is not stable in aqueous eluents above pH 8.0 [11] or where interactions with residual silanols can lead to peak tailing. At present polymeric materials tend to be used for reversed-phase separations when the separation is associated with difficulty on a silica-based packing, where chemical stability is required, such as basic compounds at high pH, or where the apparent greater hydrophobicity of the polymers can be utilized for sample pre-concentration/treatment and analysis of hydrophobic or hydrophilic species.

2.3. Macromolecule reversed-phase

Reversed-phase chromatography of the biological macromolecules, polypeptides and proteins has gained in importance in recent years. However, the analysis system involves operating at extremes of pH under gradient elution conditions and therefore requires an HPLC stationary phase which is both chemically and physically stable. Also the interaction of charged peptides and proteins with accessible residual surface silanols on a silica-based matrix can result in a mixed-mode separation and peak tailing and broadening [22]. Therefore the mechanical stability, chemical stability and lack of surface silanols makes the PLRP-S, poly(styrene–divinylbenzene) matrix a suitable alternative for reversed-phase separations of biological macromolecules. These larger molecules will be excluded from the micropores so that the reduced efficiency compared with silica-based packings experienced with small non-polar molecules as previously discussed will not be evident.

The suitability of the small pore high surface area, PLRP-S 100 Å material in the high efficiency 5 μm particle size has been demonstrated for fingerprint peptide mapping [23]. The material exhibited predictable separations with equivalent selectivity and peak capacity to that of alkyl bonded phases. Synthetic peptide purifications have also been carried out utilizing the high surface area material for maximum sample load [24]. The high selectivity of the phase was utilized for the analysis of the glycopeptide antibiotic, vancomycin, and also to monitor changes which occur during its degradation [25].

With large molecule separations where diffusion coefficiencies have been measured at 10^{-10} and 10^{-11} m^2/s [26] the pore size of the HPLC adsorbent is critical for high efficiency separations. The pore diffusion (solute transfer into and out of the porous structure of the packing material) further reduces the already low diffusion coefficients. It has been calculated that when the ratio of molecular diameter to pore diameter exceeds 0.2 the pore diffusion becomes restricted [27]. This results in a significant increase in band-spreading and hence a reduction in separation efficiency/resolution. Therefore the choice of the correct pore size PLRP-S material is critical in minimizing restricted diffusion of macromolecules. The PLRP-S 100 Å material is suitable for peptides up to approximately 15–20 amino acids in length, the 300 Å for medium-molecular-weight globular proteins, the 1000 Å for larger globular proteins or those having fibrous type structures and the 4000 Å for very large or very fast separations. Fig. 6 compares the reversed-phase separations of collagen and
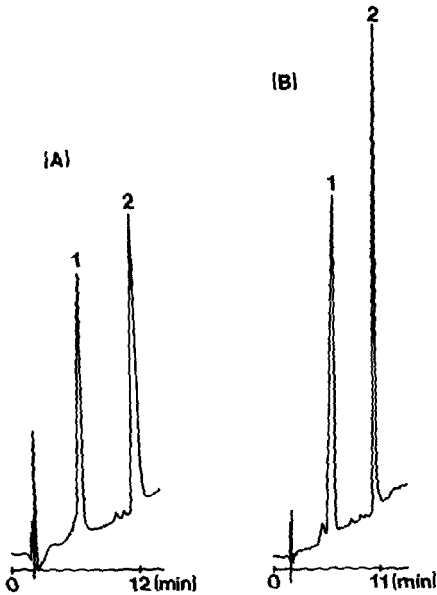
Fig. 6. Separation of fibrinogen and collagen at ambient temperature. Eluent (A) 0.25% trifluoroacetic acid in water; (B) 0.25% trifluoroacetic acid in 95% acetonitrile, 5% water; gradient, linear 20–60% B in 15 min; flow-rate, 1.0 ml/min; detector, UV operated at 220 nm. (A) Column, PLRP-S 300 Å 8 μm 150 × 4.6 mm I.D.; (B) column, PLRP-S 1000 Å 8 μm 150 × 4.6 mm I.D. 1 = Collagen; 2 = fibrinogen.

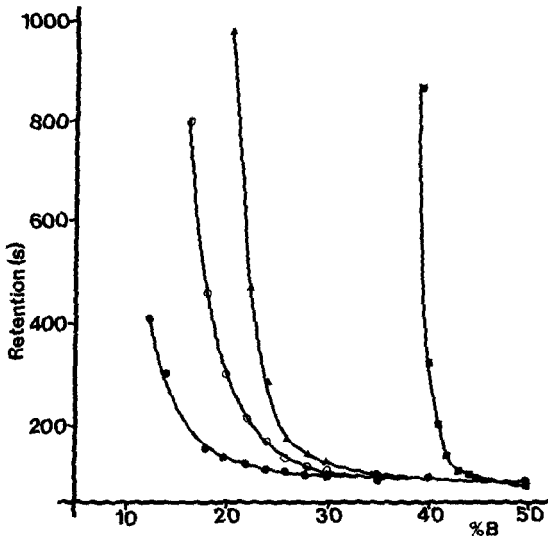Fig. 7. Plot of retention versus acetonitrile concentration for the isocratic separation of myoglobin (■), neurotensin (▲), neurotensin fragment 1–8 (●) and neurotensin fragment 8–13 (○), run on a PLRP-S 300 Å 8 μm 150 × 4.6 mm I.D. column. Eluent A, 0.1% trifluoroacetic acid in 1% acetonitrile, 99% water; eluent B, 0.1% trifluoroacetic acid in 99% acetonitrile, 1% water; flow-rate, 1.0 ml/min; detector, UV operated at 220 nm.

fibrinogen, proteins which have rod-like conformations, using the PLRP-S 300 Å and 1000 Å pore size materials. The increased band width due to restricted diffusion is clearly evident with the 300 Å material.

Proteins are known to display steep elution isotherms in reversed-phase chromatography when using silica-based packings [28]. Fig. 7 shows the plot of retention *versus* acetonitrile concentration generated using the PLRP-S 300 Å for three peptides, neurotensin, neurotensin fragment 1–8, neurotensin fragment 8–13 and the protein myoglobin. As with the silica-based packings the elution isotherm for myoglobin is steep with only a 4% solvent strength window for interaction but with the three peptides the window is considerably larger, approximately 20%.

Therefore for reversed-phase separations of proteins using the macroporous PLRP-S material the retention mechanism approaches that of an "on–off" retention model similar to the silica-based packings and therefore gradient separations are normally used. However, the smaller peptides which have adsorption isotherms similar to small molecules can be chromatographed in either an isocratic or gradient mode. When analysing the proteins it is essential for maximum efficiency/resolution that a sufficiently large pore sized HPLC adsorbent is used to minimize band broadening due to restricted diffusion within the porous matrix.

3. SURFACE COATINGS/MODIFICATIONS

3.1. Ion-exchange

The hydrophobic character of the poly(styrene–divinylbenzene) matrix is a serious limitation to its suitability for macromolecule separations other than is a reversed-phase mode. Even after derivatization the hydrophobic interactions between polymers and proteins can be sufficiently strong that the proteins may be denatured either when adsorbed onto the surface of the stationary phase or when subsequently eluted. Therefore it is essential that the hydrophobic character of the poly(styrene–divinylbenzene) matrix is masked for the chromatographic separation of biological macromolecules in all retention modes except reversed-phase.

In the same way as a silica matrix can be derivatized and/or coated to alter surface characteristics so can a polymeric support. However, when a chemically stable polymer is coated it is possible to use chemistries which are themselves exceptionally stable and so an HPLC adsorbent is produced which is capable of withstanding aggressive eluents and extremes of pH.

The polyethyleneimine coating technology, where a thin layer of the organic polyamine is adsorbed onto the surface of the silica through ion-pair formation between the silanols and amine groups and subsequent cross-linking, has been used to produce silica-based anion-exchangers for protein chromatography [29]. Subsequent studies have shown that the same coating process can be applied to negatively functionalized macroporous polymers [30,31]. The PL–SAX strong anion-exchange material has been produced by coating the PLRP-S poly(styrene–divinylbenzene) matrices which have been shown to have the required pore geometry for high efficiency separations of biological macromolecules with polyethyleneimine. This coating is then cross-linked in position to give chemical stability and quaternized to provide the strong anion-exchange functionality. The chemical stability of the matrix coupled with the quaternary amine functionality which is ionized over a broad pH range enables

TABLE 2

THE RELATIONSHIP BETWEEN PORE SIZE AND SPECIFIC IONIC CAPACITY, AS DETER-
MINED BY FRONTAL ANALYSIS, FOR THE PL–SAX RANGE OF STRONG ANION-
EXCHANGERS

Adsorbent pore size (Å)	Specific ionic capacity (mmoles +/mlCV)
100	0.51
300	0.26
1000	0.23
4000	0.21

anion-exchange separations to be performed over a wider pH range than previously possible with weak ion-exchangers based on a silica matrix. With the polymer it is possible to strip denatured protein from the column or suppress the ionization of the quaternary amine group by flushing the column with strong alkali, sodium hydroxide or acid, hydrochloric acid. As the coating is not only adsorbed onto the surface of the matrix but covalently cross-linked in position, organic modifiers can also be used in the eluent or for column clean-up.

The full range of PLRP-S adsorbents have been used as base matrices to produce polymeric strong anion-exchangers. Decreasing the adsorbent pore size increased the specific ionic capacity of the anion-exchanger as determined by frontal loading with sodium nitrate due to an increase in the total surface area, see Table 2. However, when evaluated for resolution and loading with a range of proteins of different molecular weight it was found that the small pore 100 Å had much lower loadings compared with the wider pore materials. The surface area within the pore was not accessible. The maximum loading for the smallest protein used, β-lactoglobulin, was achieved with the 300-Å pore material but unlike the 1000- and 4000-Å materials the 300-Å was unable to resolve the A and B chains. The intraparticle diffusion even with this small globular protein is very hindered resulting in excessive band broadening. Table 3 summarizes the loadings achieved with the range of proteins evaluated on each pore size. It would

TABLE 3

THE RELATIONSHIP BETWEEN PORE SIZE AND DYNAMIC PROTEIN LOAD, AS DETER-
MINED BY FRONTAL ANALYSIS, FOR THE PL–SAX RANGE OF STRONG ANION-
EXCHANGERS

Protein	Molecular weight	Dynamic protein load (mg/mlCV)			
		100 Å	300 Å	1000 Å	4000 Å
Thyroglobulin	669 000	6	10	17	23
Catalase	232 000	14	35	20	10
γ-Globulin	90 000	14	31	38	34
BSA	66 000	9	2	36	16
β-Lactoglobulin	35 000	22	108	46	20

appear that loading is related to available surface area with the PL–SAX 1000 Å material being optimum for the medium-molecular-weight globular proteins and the PL–SAX 4000 Å material for the larger solutes such as thyroglobulin. The anomaly with catalase is due to it denaturing to sub-units of 57 500 molecular weight.

Therefore for the analysis or purification of small/medium molecular weight proteins maximum loading coupled with good resolution would be achieved with the PL–SAX 1000 Å material but for the analysis of larger molecules such as thyroglobulin or polynucleotides then the wider pore PL–SAX 4000 Å would be optimum.

In addition to the PL–SAX strong anion-exchanger a strong cation-exchange material has been produced, PL–SCX, with sulphonic acid functionalities and a masked hydrophobic surface. With ion-exchange materials which are often used for preparative fractionations on a mg to kg scale in addition to analytical separations the two wide pore sized materials, PL–SCX 1000 Å and PL–SCX 4000 Å, have been produced. As approximately 95% of the surface area of a porous adsorbent is located within the pore structure of the matrix it is essential that for high capacity the pores are sufficiently large to give free access to the internal surface area. As with the strong anion-exchanger the derivatization chemistries are covalent and therefore the PL–SCX matrix is able to withstand operation at extremes of pH, with high salt eluents and organic modifiers. The modifications carried out to the base poly(styrene–divinyl-benzene) matrix to produce the ion-exchangers do not weaken the polymer or reduce mechanical stability so enabling high pressures and flow-rates to be used as with the reversed-phase materials.

3.2. Gel filtration

For gel filtration chromatography (GFC) of biological molecules which has traditionally been carried out on polyacrylamide and polysaccharide soft gels or more recently diol coated silica matrices, surface hydrophilicity, homogeneity and reproducibility are critical. Although the low cross-linked polyacrylamides and polysaccharides exhibit minimal non-specific interactions with a range of sample types the inherent softness of the polymers limits their suitability for high speed separations. The silica-based GFC packings have shallow calibration curves over a specific molecular size range for high resolution separations [32] but are limited due to lack of chemical stability, residual silanols that can function as cation-exchange groups, resulting in non-specific interactions, and increasing fragility as the pore size increases [33]. The chemistries used to produce the PL–GFC range of gel filtration columns produces an exceptionally hydrophilic surface with minimum ion-exchange functionalities.

In order to probe the surface homogeneity of the PL–GFC matrix four proteins of different hydrophobicity and charge were used as chromatographic test probes myoglobin with an isoelectric point of 6.76–7.16, ovalbumin with an isoelectric point of 4.6, α-chymotrypsinogen A at 9.1 and lysozyme at 11.0. When chromatographed at pH 7 ovalbumin would have a nett negative charge, lysozyme and α-chymotrypsinogen A a nett positive charge and myoglobin would be neutral. By measuring the retention volume of these four proteins in a low salt buffer with increasing concentrations of salt, ammonium sulphate, it is possible to probe the surface for both electrostatic functionalities and hydrophobicity. Fig. 8 shows the plots of retention *versus* ammonium sulphate concentration for the four proteins chromatographed on the three individual pore sizes, PL–GFC 300, 1000 and 4000 Å. From these PL–GFC
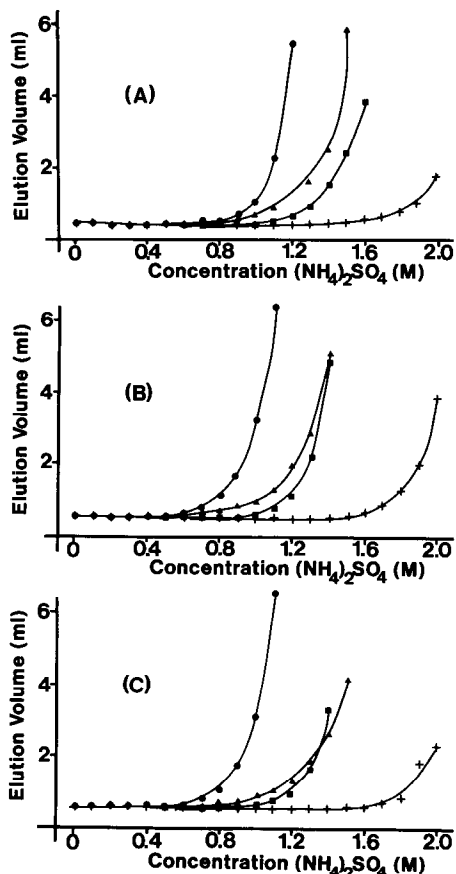
Fig. 8. Plot of elution volume *versus* concentration of $(NH_4)_2SO_4$ in 0.02 M KH_2PO_4 at pH 7.0 for the proteins α-chymotrypsinogen A (●), lysozyme (▲), ovalbumin (■) and myoglobin (+), run on PL–GFC 300 Å (A), PL–GFC 1000 Å (B) and PL GFC 4000 Å (C). Column, 50 × 4.6 mm I.D. 8 μm; flow-rate, 1.0 ml/min; detector, UV operated at 220 nm.

salt plots it is evident that there is no increase in retention at low salt concentrations indicating minimal non-specific interactions due to either cation- or anion-exchange functionalities and that hydrophobic interactions are not promoted even with α-chymotrypsinogen A until the ammonium sulphate concentration reaches 0.5 M. There is therefore a wide operating window of ionic strength where size separations could be expected to dominate making the material suitable for gel filtration chromatography of a wide range of water soluble polymers, neutral, cationic, anionic and hydrophobic.

Three individual pore sizes have been produced, PL–GFC 300 Å with a pullulan polysaccharide resolving range of 100 to 200 kDa, PL–GFC 1000 Å which resolves from 20 kDa to 2000 kDa and the PL–GFC 4000 Å resolving from 100 kDa to greater than 20 000 kDa. The materials have optimized pore size distributions to be used

individually or in the case of the 300 and 4000 Å where there is a small overlap in the resolving ranges, in series for the analysis of broad distribution polymers.

As with the previous polymeric materials discussed, the PL–GFC polymers are chemically stable enabling gel filtration chromatography to be carried out over a wide pH range with acid, alkali and methanol used as eluents, additives or in clean-up cycles without altering the surface characteristics.

3.3. Affinity chromatography

A survey undertaken in 1986 found that affinity chromatography (AFC) was the second most widely used purification procedure after ion-exchange [34]. This is due to the unique biological affinity between bound ligand and solute which confers exceptionally high selectivity on the separation mode. It is often possible to separate one or a specific type of solute from hundreds of unretained species. However, as it is a separation mode often used as part of a purification process the demands on the packing material are high. It is essential that the ligand does not leach from the packing material or that the matrix itself does not degrade or dissolve to contaminate the product. Also it is essential as with gel filtration chromatography that all non-specific binding is minimized if high purity is to be achieved. For these reasons a chemically stable polymer matrix which can be derivatized for covalent attachment of a ligand and which can be operated at high flow-rates to take advantage of the rapid bind/elute kinetics of affinity separations would be the ideal base material for an affinity HPLC adsorbent.

The PL–AFC matrix is such a rigid chemically stable polymer which has a hydrophilic surface to minimize non-specific interactions and polyhydroxyl function-alities which can be derivatized for ligand attachment. Non-pyrogenic protein A isolated from *Staphylococcus aureus* has been covalently attached to the surface of the PL–AFC matrix to produce an affinity material with a high specificity for the Fc region of certain immunoglobulins (antibodies). The PL–AFC Protein A material can be used to purify IgG, to selectively remove IgG or to isolate immunocomplexes for antigen purification.

3.4. High speed separations

There is continued interest in increasing the speed of analysis in the separation/fractionation of biological macromolecules. In 1967, Horváth made pellicular column packings by coating solid glass beads to produce an anion-exchanger so eliminating stagnant mobile phase mass transfer which leads to band-broadening and hence a loss in efficiency and resolution [35]. However, these materials were limited due to the large particle size and hence low sample capacity. More recently with the availability of microparticulate, 1.5 μm, non-porous silica packings interest has again been stimulated in the use of pellicular packings for high speed separations including affinity [36] reversed-phase [37] hydrophobic interaction [38] and larger particle sized polymers for ion-exchange [39]. However, even with the smaller particle sized material the capacity is still low and the columns have a greater tendency to block and suffer from reduced permeability [40].

An alternative approach to improving the speed of analysis of biological macromolecules whilst maintaining sample capacity would be to use a rigid macroporous polymer with an exceptionally open porous structure. The PLRP-S
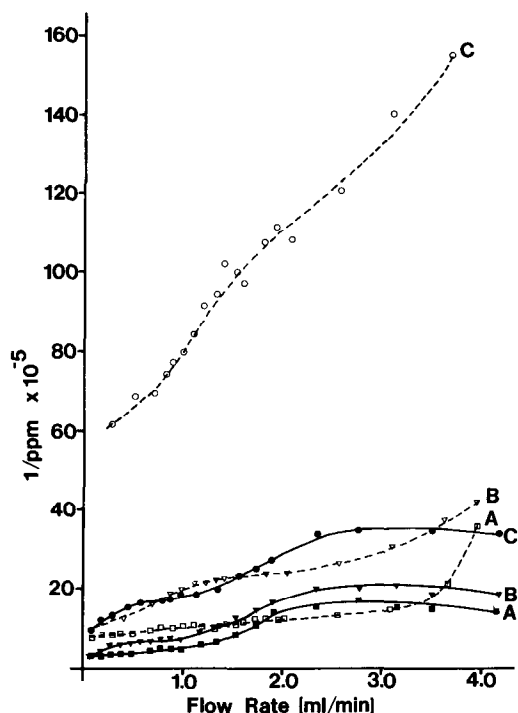
Fig. 9. Plot of reciprocal of column efficiency *versus* flow-rate for adenosine 5′-monophosphate (A), myoglobin (B) and ferritin (C) run on PL–SAX 4000 Å 8 μm (————) and PL–SAX 1000 Å 8 μm (- - -), in a non-interactive mode. Column, 250 × 4.6 mm I.D.; eluent, 0.01 *M* Tris–HCl, 0.5 *M* NaCl, pH 8.0; detector, UV operated at 280 nm.

4000 Å, PL–SAX 4000 Å and PL–SCX 4000 Å materials have been evaluated for their suitability [41].

In order to compare the dependence of efficiency on flow-rate for large molecules the PL–SAX 1000 Å and 4000 Å materials were run isocratically with three probes adenosine 5′-monophosphate, myoglobin and ferritin in a high salt buffer, *i.e.*, non-interactive conditions. Plate count measurements to determine efficiency were carried out at various flow-rates. Fig. 9 is the plot of the reciprocal of efficiency *versus* flow-rate the test probes run on the two pore sizes. As expected with the PL–SAX 1000 Å material at a given flow-rate there is a decrease in efficiency with increasing size and a more significant decrease in efficiency with increasing flow-rate for the large molecules, myoglobin molecular weight of 16 950 daltons and ferritin 470 000 daltons. This is due to the larger molecular diffusion coefficients for the bigger molecules and increased hindrance to intraparticle mass transfer at high flow-rates. However, with the PL–SAX 4000 Å material, although there is a decrease in efficiency with increased molecular size at a given flow-rate, as the flow-rate increases the decrease in efficiency is not so marked and at a critical flow-rate is halted. It would appear that there is a critical linear velocity irrespective of solute size, approximately 3.5 mm/s, above which no further reduction in efficiency occurs for the 4000-Å pore material. This type

of curve is not typical for porous materials although it has been observed with non-porous packings. However, the dynamic sample capacity of the material does not change, for the PL–SAX 4000 Å being 34 mg BSA/ml at 1.0 ml/min and 32 mg BSA/ml at 4.0 ml/min on a 50 × 4.6 mm I.D. column, the shape of the breakthrough curve is unaltered. Therefore the 4000-Å pore size materials can be operated with steep gradients at high flow-rates without a loss in resolution. Typically separations of complex mixtures can be achieved within two minutes with resolution comparable to or better than the normal 20–30 min gradient runs and with comparable sample capacities.

4. CONCLUSIONS

The development of microparticulate macroporous polymers, such as the poly(styrene–divinylbenzene), which have improved mechanical strength compared with the soft microporous classical liquid chromatography gels now enables separations to be achieved in times comparable to or better than those obtained with the inorganic silica based matrices but with chemically stable polymers. Stationary phases with similar functionalities and selectivity to those of soft gels and silicas can be produced by surface coatings and/or derivatization.

In small molecule reversed-phase chromatography the polymer based packings are utilized in preference to silica based packings when increased chemical stability is required or when silanol effects need to be eliminated. There is some difference in selectivity due to the possibility of π–π or charge transfer interactions. The highly "effective" hydrophobicity makes the small pore, PLRP-S 100 Å, material suitable for sample preconcentration/treatment applications where it is necessary to minimize sample loss during washing cycles and for the analysis of very hydrophilic or hydrophobic species. The excellent chemical stability enables operation at extremes of pH for ion suppression or where detection can be improved by inducing fluorescence. It is important if maximum efficiency is to be achieved, that consideration is given to the choice of organic solvent used in the mobile phase and the temperature at which the column is operated.

There are distinct advantages to the use of these rigid macroporous polymers for the separation of biological molecules where harsh elution conditions or column clean-up cycles are employed. The chemical stability and covalent attachment of functionalities enable purifications to be achieved without the risk of product contamination due to either leached ligands or dissolved support matrix.

Through the optimization of pore size and pore size distribution and improved mechanical strength high speed, high efficiency separations of biological macromolecules can be achieved with high sample capacity.

The 4000-Å pore size material can be operated with steep gradients at high flow-rates for rapid fractionation or separation of large biomolecules but without the loss in efficiency due to reduced intraparticle diffusion experienced with conventional HPLC adsorbents.

Using the new generation of mechanically stable macroporous HPLC supports high efficiency, high speed separations are now achievable with chemically stable packings.

REFERENCES

1 H. A. Sober and E. A. Peterson, *J. Am. Chem. Soc.*, 76 (1954) 1711.
2 E. A. Peterson, *Cellulosic Ion Exchangers*, Elsevier, Amsterdam, 1970.
3 J. Porath and P. Flodin, *Nature (London)*, 183 (1959) 1657.
4 S. Moore, *J. Biol. Chem.*, 243 (1968) 6281.
5 H. D. Scobell and K. M. Brobst, *J. Chromatogr.*, 212 (1981) 51.
6 J. C. Moore, *J. Polym. Sci., Part A*, 2 (1964) 835.
7 J. V. Dawkins, T. Stone, G. Yeadon and F. P. Warner, *Polymer*, 20 (1979) 1164.
8 J. V. Dawkins, L. L. Lloyd and F. P. Warner, *J. Chromatogr.*, 352 (1986) 157.
9 D. C. Sherrington, in P. Hodge and D. C. Sherrington (Editors), *Polymer Supported Reactions in Organic Synthesis*, Wiley, Chichester, 1980, ch. 1.
10 J. V. Dawkins and M. Heming, *Makromol. Chem.*, 176 (1975) 1795.
11 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, 1979.
12 N. Tanaka, K. Hashizume, M. Araki, H. Tsuchiya, A. Okuno, K. Iwaguchi, S. Ohnishi and N. Takai, *J. Chromatogr.*, 448 (1988) 95.
13 T. N. Tweeton, L. L. Lloyd and F. P. Warner, *36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy, New Orleans, MI, February 25–March 1, 1985*, Abstract No. 309.
14 P. J. Rennie and S. F. Mitchell, *Chromatographia*, 24 (1987) 319.
15 L. L. Lloyd, F. P. Warner, J. F. Kennedy and C. A. White, *J. Chromatogr.*, 437 (1988) 447.
16 J. D. Berg, R. A. C. Allen Narket, C. V. H. Bradby and B. M. Buckly, *Ann. Clin. Biochem.*, 25 (1988) 815.
17 R. M. Smith, *J. Chromatogr.*, 291 (1984) 372.
18 J. L. Robinson, W. J. Robinson, M. A. Marshall, A. D. Barnes, K. J. Johnson and D. S. Salas, *J. Chromatogr.*, 189 (1980) 145.
19 M. Popl, V. Dolansky and J. Fahrnich, *J. Chromatogr.*, 148 (1978) 195.
20 H. M. Stuurman, J. Kohler, S. O. Jansson and A. Litzen, *Chromatographia*, 23(5) (1987) 341.
21 L. D. Bowers and S. Pedigo, *J. Chromatogr.*, 371 (1986) 243.
22 H. Engelhardt and H. Muller, *Chromatographia*, 19 (1984) 19.
23 J. K. Swadesh, *J. Chromatogr.*, 512 (1990) 315.
24 L. L. Lloyd and F. P. Warner, *14th International Symposium on Column Liquid Chromatography, Boston, MA, May 20–25, 1990*, Abstract No. 643.
25 A. H. Thomas and P. Newland, *J. Chromatogr.*, 410 (1987) 373.
26 G. Guiochon and M. Martin, *J. Chromatogr.*, 326 (1985) 3.
27 K. K. Unger, R. Janzen and G. Jilge, *Chromatographia*, 24 (1987) 144.
28 E. C. Nice, M. N. Capp, N. H. C. Cooke and M. J. O'Hare, *J. Chromatogr.*, 218 (1981) 569.
29 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375.
30 N. Kopaciewicz, *Ph.D. Dissertation*, Purdue University, West Lafayette, IN, 1984.
31 M. A. Rounds, W. D. Rounds and F. E. Regnier, *J. Chromatogr.*, 397 (1987) 25.
32 E. Pfannkock, K. C. Lu, F. E. Regnier and H. Barth, *J. Chromatogr.*, 18 (1980) 430.
33 E. Engelhardt and D. Mathes, *J. Chromatogr.*, 142 (1977) 311.
34 J. Bannerjea, S. Oh, M. Hoare and P. Dunhill, *Biotechnology*, 4 (1986) 954.
35 Cs. Horváth, B. A. Preiss and S. R. Lipsky, *Anal. Chem.*, 39 (1967) 1422.
36 B. Anspach, K. K. Unger, H. Giesche and M. T. W. Hearn, *4th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baltimore, MD, December, 1984*, Abstract No. 103.
37 K. K. Unger, G. Jilge, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 359 (1986) 61.
38 R. Janzen, K. K. Unger, H. Giesche, J. N. Kinkel and M. T. W. Hearn, *J. Chromatogr.*, 397 (1987) 91.
39 M. A. Rounds and F. E. Regnier, *J. Chromatogr.*, 443 (1988) 73.
40 G. P. Rozing and H. Goetz, *J. Chromatogr.*, 476 (1989) 3.
41 L. L. Lloyd and F. P. Warner, *J. Chromatogr.*, 512 (1990) 365.