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Polymer-coated reversed-phase packings with controlled hydrophobic properties

I. Effect on the selectivity of protein separations

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ABSTRACT

A novel approach to analytical and preparative protein separations by reversed-phase chromatography is described. Porous and non-porous silica supports were coated with polymethacrylate-based polymers or copolymers to produce tailored stationary phases of varying hydrophobicity. Through the application of a model protein mixture of lysozyme, cytochrome *c* and myoglobin, it was demonstrated that selective unfolding of proteins can be achieved by varying the hydrophobicity of the polymer coat permitting manipulation of the chromatographic pattern of analytical protein separations. Thus, proteins may be maintained in their native, folded state or may be partially or completely unfolded, depending on the choice of packing and/or run conditions. In addition, on the non-porous packings, such manipulation of protein elution profiles is achievable at run times of <5 min. A potential preparative role for the polymer-coated packings was demonstrated through their application to the reversed-phase chromatography of a three-protein complex, rabbit skeletal troponin. Through packing and/or temperature manipulation, it was demonstrated that even such a multiprotein complex, stabilized only by non-covalent interactions, may be maintained during purification by reversed-phase chromatography.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has seen a significant increase in recent years in its application to polypeptides and proteins [1,2]. However, its use in this regard still does not rival the extent of its application to smaller peptide molecules (<50 residues). Reasons for many researchers' reluctance to utilize RP-HPLC for protein separations have included such concerns as protein denaturation, poor recoveries, broad misshapen peaks, ghosting and loss of activity with biologically active proteins such as enzymes [3].

The most commonly used solvent systems for RP-HPLC of polypeptides involve linear increasing gradients, starting with water and increasing concentrations of organic solvent (usually methanol, acetonitrile or isopropanol) [1,2]. These solvent systems generally employ low concentrations of perfluorinated organic acids [*e.g.*, trifluoroacetic acid (TFA)] at a concentation of 0.05–0.1% (v/v) in both the water and the organic solvent, resulting in a pH of *ca.* 2.0. Although many proteins are susceptible to unfolding in such aqueous organic solutions at acidic pH, particularly during prolonged exposure, Lau *et al.* [4] demonstrated that the primary cause of protein unfolding during RP-HPLC is the hydro-

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phobicity of the stationary phase which disrupts the hydrophobic interactions stabilizing the native conformation.

RP-HPLC separations of polypeptides have generally been carried out on silica-based matrices containing alkyl (*e.g.*, C_3, C_4, C_8, C_{18}) hydrophobic functional ligands. As hydrophobic interactions play a major role in stabilizing the three-dimensional structure of a protein, it is not surprising that the hydrophobicity of such matrices (especially considering the relatively high hydrocarbon loadings typical of such stationary phases) could unfold a protein on binding to the column. As pointed out by Lau *et al.* [4], this may preclude the purification of multi-subunit proteins or of any proteins where a separation in the native conformation is desired.

The development of hydrophobic interaction chromatography (HIC), another separation method base on hydrophobic interactions between the solute and the stationary phase, was spurred by the tendency of proteins to become unfolded during RP-HPLC [5–7]. For HIC, ligands of lower hydrophobicity, lower ligand densities and aqueous solutions (absence of organic modifier) at neutral pH are used, all with the intention of maintaining a protein in its native conformation. In spite of the milder conditions of this technique compared with RP-HPLC, changes in protein tertiary and/or quaternary structure (the stability of which are heavily dependent on hydrophobic interactions), during HIC cannot be ruled out [7]. In addition, the presence of high concentrations of stabilizing salts characteristic of HIC may necessitate a subsequent desalting step prior to further characterization or application.

A major advantage of RP-HPLC, apart from its powerful resolving capability, is the availability of volatile mobile phases, such as the frequently employed aqueous TFA to TFA–acetonitrile gradient elution system. The utility of such a system combined with maintenance of the native state of a protein is clear. In addition, as proteins vary in their degree of stability, packings with various degrees of hydrophobicity would be extremely useful in protein separations by affecting selectivity due to differential induction of conformational change. Indeed, several researchers [7–11] have observed that changes in sorbent hydrophobicity and/or temperature could be used to improve the resolution of proteins with similar retention times through controlled unfolding of proteins in a protein mixture. However, there are practical limits to decreasing the ligand density of silica-based packings containing functional ligands such as alkyl or phenyl moieties [7], since the effectiveness and capacity of the column would decrease simultaneously.

There have been major advances in recent years in the design and development of stationary phases for RP-HPLC, frequently with a focus on novel concepts and improvement of the stationary phase chemistry of RP-HPLC packings [12,13]. One approach has been to immobilize defined polymer layers at the surface of rigid non-porous inorganic or porous inorganic supports in such a way that a solute impermeable layer results [13,14]. The approach described in this paper involved coating porous or non-porous silica supports with polymethacrylate-based polymers or copolymers, such materials lending themselves well for the preparation of stationary phases of controlled hydrophobicity. Employment of monodisperse non-porous silica particles enabled us to take advantage of the rapid analysis times and high recovery of biopolymers typical of such packings, because with such micropellicular sorbents the chromatographic interactions are limited to the outer surface [14–17]. The more commonly employed porous silica support, in addition to its greater sample capacity compared with non-porous silica, also serves as a useful comparison with the micropellicular packings. From the observed RP-HPLC chromatographic profiles of protein mixtures, we have demonstrated the potential value of the novel concept of employing stationary phases with a range of hydrophobicities for both analytical and preparative protein separations.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile were obtained from J. T. Baker (Phillipsburg, NJ, USA) and HPLC-grade TFA from Pierce (Rockford, IL, USA). Cyclohexanol, toluene, sodium perchlorate and ammonium sulphate were obtained from Fisher (Fairlawn, NJ, USA). 1,4-Dioxane and sodium chloride were obtained from BDH (Vancouver, BC, Canada). *n*-Pentane was obtained from E. Merck (Darmstadt, Germany), methanol from E. Merck

and J. T. Baker and dicumyl peroxide (DCP) from Aldrich Chemie (Steinheim, Germany). Equine cytochrome *c*, chicken lysozyme and equine myoglobin were obtained from Sigma (St. Louis, MO, USA). Rabbit skeletal whole troponin, troponin C (TnC), troponin I (TnI) and troponin T (TnT) were prepared from tissue extracts in the laboratory of R. S. Hodges.

Apparatus

The HPLC instrument consisted of a Hewlett-Packard (Avondale, PA, USA) HP1090 liquid chromatograph, coupled to an HP1040A detection system, HP9000 Series 300 computer, HP9133 disc drive, HP2225A Thinkjet printer and HP7440A plotter. Columns were packed by means of a Shandon (Sewickley, PA, USA) or a Haskel (Burbank, CA, USA) packing pump.

Supports

Monospher (non-porous silica; 1.7-μm mean particle diameter) and LiChrospher (porous silica; 10 μm; 300-Å pore size) supports were obtained from E. Merck.

Preparation of packings

Preparation of silicas. Pretreatment of the non-porous Monospher support was carried out as described by Hanson *et al.* [13]. The porous LiChrospher support did not require prepreparation.

Preparation of prepolymer of poly(2-hydroxyethyl methacrylate) (P2HEMA). A mixture of 2-hydroxyethyl methacrylate (2-OH-EMA) (20 g) and DCP (0.25 g), the radical starter, was heated under reflux at 100°C for 75 min and cooled, producing oligo-2-OH-EMA, a prepolymer soluble in methanol.

Preparation of precopolymer of ethyl methacrylate–2-hydroxyethyl methacrylate (P2HE-E) copolymer. A mixture of ethyl methacrylate (EMA) (10 g), 2-OH-EMA (10 g) and DCP (0.25 g) was heated under reflux at 100°C for 75 min and cooled, producing oligo-EMA–2-OH-EMA, a precopolymer soluble in methanol.

Preparation of prepolymer of poly(ethyl methacrylate) (PEMA). A mixture of EMA (20 g) and DCP (0.25 g) was heated under reflex at 100°C for 2 h and cooled, producing oligo-EMA, a prepolymer soluble in both diethyl ether and toluene.

Preparation of precopolymer of octadecylmethacrylate–methylmethacrylate copolymer (POMA). Octadecyl methacrylate (OMA) (10 g), a solid at room temperature, was dissolved together with DCP (0.25 g) in methyl methacrylate (MMA) (10 g), heated under reflux for 3 h and cooled. The resulting oligo-OMA–MMA copolymer (25% OMA–75% MMA) was soluble in *n*-pentane.

Coating of silica supports

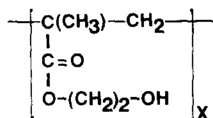
Monospher. Monospher beads (30 g) were added to a solution of prepolymer (0.3 g) and DCP (15 mg) in 50 ml of the relevant solvent.

LiChrospher. LiChrospher beads (15 g) were added to a solution of prepolymer (1.5 g) and DCP (76 mg) in 50 ml of the relevant solvent.

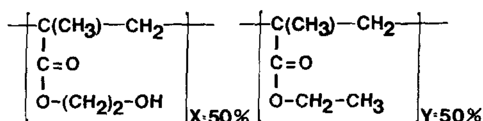
Following evaporation of the solvent, the coating procedure was carried out as described by Hanson *et al.* [13].

The formulae of the immobilized polymers and copolymers are shown in Fig. 1. Relative hydro-

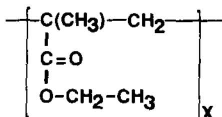
P2HEMA



P2HE -E



PEMA



POMA

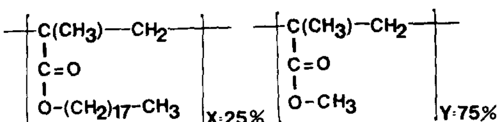


Fig. 1. Structures of methacrylate-based stationary phases synthesized in this study.

phobicities of these packings are P2HEMA < P2HE-E < PEMA < POMA. It should be stressed that these procedures produce stationary phases where the polymers and copolymers are adsorbed on the surface of the supports and are not covalently linked to the silicas in any way.

Clean-up procedure

Monospher. The polymers and copolymer-loaded Monospher supports were cleaned as described by Hanson *et al.* [13].

LiChrospher. The coated LiChrospher supports were subjected to a sedimentation process in toluene–1,4-dioxane, followed by the procedure described by Hanson *et al.* [13] for Monospher packings, except for the elimination of a sonication step.

Column packing

Monospher. Monospher packings were suspended in 1,4-dioxane–toluene–cyclohexanol (1:1:1, v/v/v) and packed by the downward flow method into stainless-steel columns (36 × 4.6 mm I.D.) (Bischoff, Leonberg, Germany) at a packing pressure of 600 bar, using methanol as packing solvent.

LiChrospher. LiChrospher packings were packed identically with the Monospher packings, except that a packing pressure of 350 bar was used.

RESULTS AND DISCUSSION

The reversed-phase packings described above were applied to the separation of two groups of proteins:

(1) Lysozyme, cytochrome *c* and myoglobin: this mixture of monomeric proteins provides a criterion for evaluating the effects of different column packings on changes in protein tertiary structure. These three proteins have frequently been utilized for such purposes [7,10,11]. Myoglobin is particularly useful for demonstrating solvent and/or stationary phase effects on tertiary structure due to the presence of a non-covalently bound haeme group (detectable at 400 nm). In contrast, the haeme of cytochrome *c* is covalently bound to the polypeptide portion of the molecule. Lysozyme is also an ideal protein for these studies as it exists only in two forms (unfolded or native) during chromatography. In addition, they serve to highlight the utility of polymethacrylate-coated silicas for analytical protein separations through selective protein unfolding.

(2) Rabbit skeletal troponin (Tn), consisting of troponin T (TnT), troponin I (TnI) and troponin C (TnC): this thin filament muscle protein, containing three protein subunits, permits the evaluation of the effects of column packings on the chromatographic behaviour of a multi-protein complex stabilized only by non-covalent interactions. In addition, they illustrate potential preparative applications of polymethacrylate-coated silica.

Application of polymethacrylate-coated silicas to analytical protein separations

Fig. 2 shows the changes in the elution profile of the mixture of lysozyme, cytochrome *c* and myoglobin on non-porous (Monospher), polymer-coated packings of varying hydrophobicity (P2HEMA < PEMA < POMA; Fig. 2A, B and C, respectively). The protein mixture was dissolved in water instead of the starting eluent of 0.05% aqueous TFA (pH 2). Although it has been demonstrated that neither lysozyme nor myoglobin is unfolded in 0.1% aqueous TFA [7,11], it was felt that the stability of even these two proteins would be better assured in the absence of acid prior to their injection on the columns.

From Fig. 2, there is a dramatic effect of packing hydrophobicity on the chromatographic profile of the protein mixture, as evidenced by the change in protein elution order as the relative hydrophobicity of the packing is increased. The elution order on the least hydrophobic packing, P2HEMA (Fig. 2A), is native lysozyme (Ln) followed by cytochrome *c* and myoglobin. As the hydrophobicity of the packing is increased to PEMA (Fig. 2B), although the same basic elution order as that observed on P2HEMA is maintained, there is now the appearance of the denatured (unfolded) lysozyme (Ld) peak between cytochrome *c* and myoglobin. As the packing hydrophobicity is increased further to POMA (Fig. 2C), the elution order is now cytochrome *c*, lysozyme (Ld) and myoglobin, with no native lysozyme remaining in the elution profile. On the P2HEMA packing, lysozyme is maintained in its native (folded) state. The surface of a protein is considerably less hydrophobic than its interior, where the majority of hydrophobic residues are located, hence the native lysozyme molecule is eluted prior to the unfolded molecule, where all formerly interior residues are now fully exposed. A similar switch in elu-

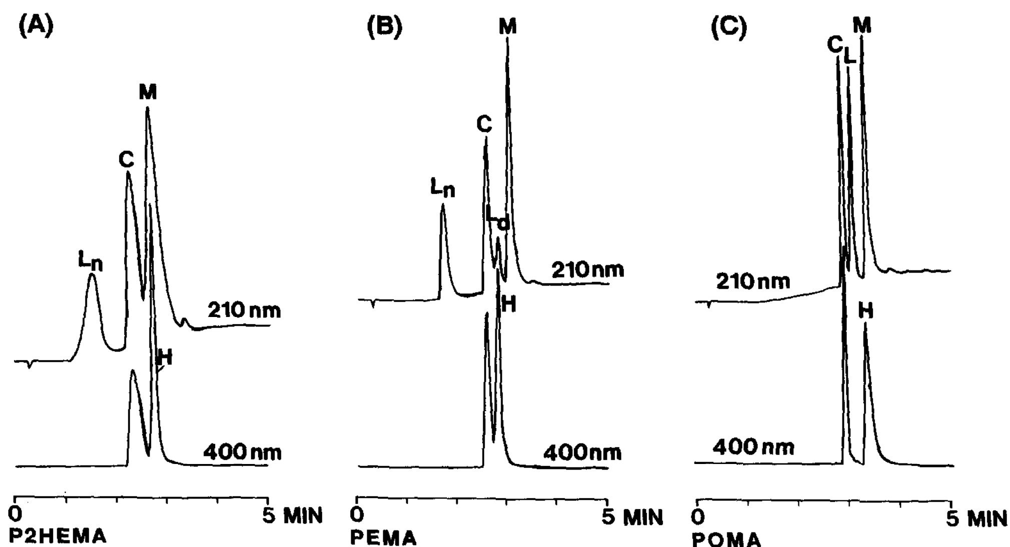


Fig. 2. Effect of hydrophobicity of non-porous Monospher packings on RP-HPLC elution profiles of proteins. (A), (B) and (C) show the elution profiles obtained on the P2HEMA, PEMA and POMA packings, respectively. Mobile phase, linear A–B gradient (20% acetonitrile/min) at a flow-rate of 1 ml/min, where A is 0.05% aqueous TFA and B is 0.05% TFA in acetonitrile; temperature, 25°C. Ln, Ld, C, M and H denote, native lysozyme, denatured lysozyme, cytochrome *c*, myoglobin and haeme, respectively. Sample mixture dissolved in water.

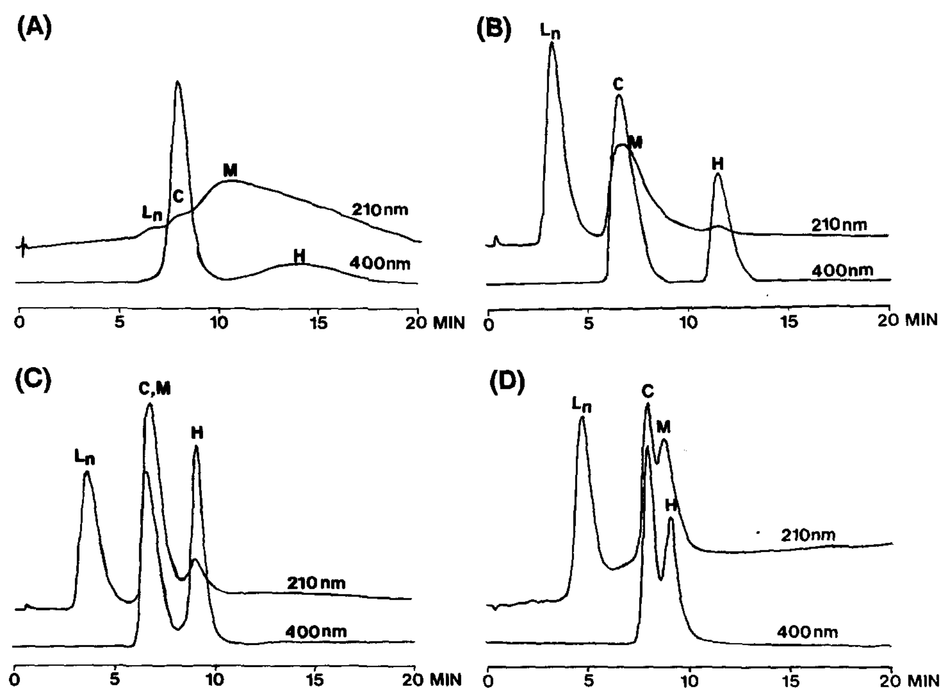


Fig. 3. RPC of proteins on porous LiChrospher P2HEMA packing. Mobile phase: (A) linear A–B gradient (4% acetonitrile/min) at a flow-rate of 1 ml/min, where A is 0.05% aqueous TFA and B is 0.05% TFA in acetonitrile; (B), (C) and (D), linear A–B gradient (4% acetonitrile/min) at a flow-rate of 1 ml/min, where A is 0.05% aqueous TFA and B is 0.05% TFA in 50% aqueous acetonitrile (B), 60% aqueous acetonitrile (C) or 80% aqueous acetonitrile (D), both solvents containing 0.1 *M* $(NH_4)_2SO_4$ (B), 0.1 *M* NaCl (C) or 0.1 *M* $NaClO_4$ (D); temperature, 25°C. The different proportions of acetonitrile to water in solvent B are due to the different solubilities of the various salts. Ln, C, M and H denote lysozyme, cytochrome *c*, myoglobin and haeme, respectively. Sample mixture dissolved in water.

tion order of these three proteins was reported by Mant and Hodges [11] when comparing two silica-based hydrophobic interaction packings differing only in the ligand density of the phenyl functional groups.

The conformational states of cytochrome *c* and myoglobin on the P2HEMA packing cannot be confirmed. The sharpness of the haeme peak of myoglobin compared with the broader myoglobin peak (in contrast to cytochrome *c*, where the protein and haeme peaks are very similar) suggests that this non-covalently bound group may be acting independently from the polypeptide chain, *i.e.*, although the haeme group and the polypeptide portion of myoglobin (apomyoglobin) are co-eluted on the P2HEMA packing under the conditions employed, the myoglobin has probably been either partially or totally unfolded. The different retention times of the haeme group and apomyoglobin on the PEMA and POMA packings indicate unfolding of myoglobin on these more hydrophobic stationary phases.

Fig. 3 illustrates the effect of salt on the chromatographic behaviour of the three proteins on the porous LiChrospher P2HEMA packing. The satisfactory performance of the Monospher P2HEMA packing (Fig. 2A) in separating the three proteins indicated that the responsibility for the poor elution profile shown in Fig. 3A (obtained by a conventional aqueous TFA to TFA–acetonitrile gradient) may lie with the porous LiChrospher support rather than the methacrylate polymer coating. Badly tailing and skewed peaks are frequently the result of non-ideal reversed-phase column behaviour in the form of electrostatic, in addition to hydrophobic, solute–packing interactions. It is well known that underivatized and ionized (negatively charged) silanols on conventional silica-based packings (e.g., alkyl- or phenyl-bonded packings) may cause such non-ideal behaviour [1,2,18]. Even though the low pH (pH 2) of TFA-based mobile phases should ensure protonation of these silanols, this is not always the case [18]. In this study, the silanols on the silica supports were not covalently linked to a functional ligand. Instead, the supports were coated with methacrylate-based polymers. With such packings, it is known that polymer film thickness will affect packing hydrophobicity [13]. The film thicknesses (and carbon coverage/m^2) of the packings prepared in this study were minimized to ensure that the individual stationary phases were not too hydrophobic, as selective protein denaturation was the goal. Owing to the synthesis process, a minimum of *ca.* 1% (w/w) of polymer is required to produce a satisfactory coating on the non-porous Monospher support. As a compromise, for the porous LiChrospher material, 10% (w/w) of polymer was employed during synthesis, with a concomitant loss of some polymer during the synthesis process. This results in a higher relative overall coverage of the Monospher support compared with that of the LiChrospher silica, as the non-porous particles have a much lower interactive surface area compared with the porous silica. Hence it is possible that, whereas the polymer coverage of the Monospher particles was sufficient to prevent potential silanol problems, this was not the case with the porous LiChrospher support.

The addition of almost any salt to the mobile phase will suppress undesired electrostatic solute–packing interactions during RP-HPLC, thereby improving the chromatographic profile [1,2,19]. Fig 3B, C and D show the effects on the protein chromatographic profiles of adding ammonium sulphate, sodium chloride and sodium perchlorate respectively, (all at a 0.1 *M* concentration) to the mobile phase. Although the presence of all three salts produced improved the chromatographic profiles over those obtained in their absence (Fig. 3A), their effectiveness varied considerably. Thus, there was improved peak shape on substituting sodium chloride (Fig. 3C) for ammonium sulphate (Fig. 3B). Whereas lysozyme remains in its native state (Ln), as observed previously on the Monospher P2HEMA packing (Fig. 2A), myoglobin is clearly unfolded, with its haeme group eluted independently of apomyoglobin. Cytochrome *c* and apomyoglobin are co-eluted in the presence of either of these two salts. Not only is there a further improvement in protein peak shape with the addition of sodium perchlorate to the mobile phase (Fig. 3D), but cytochrome *c* and myoglobin are now partly resolved, resulting in the same elution order as that observed on the Monospher P2HEMA packing (Fig. 2A).

Fig. 4 shows the chromatographic profiles of the protein mixture obtained on LiChrospher packings of increasing hydrophobicity (P2HEMA < P2HE-E < PEMA < POMA; Fig. 4A, B, C and D, respectively) in the presence of 0.1 *M* sodium per-

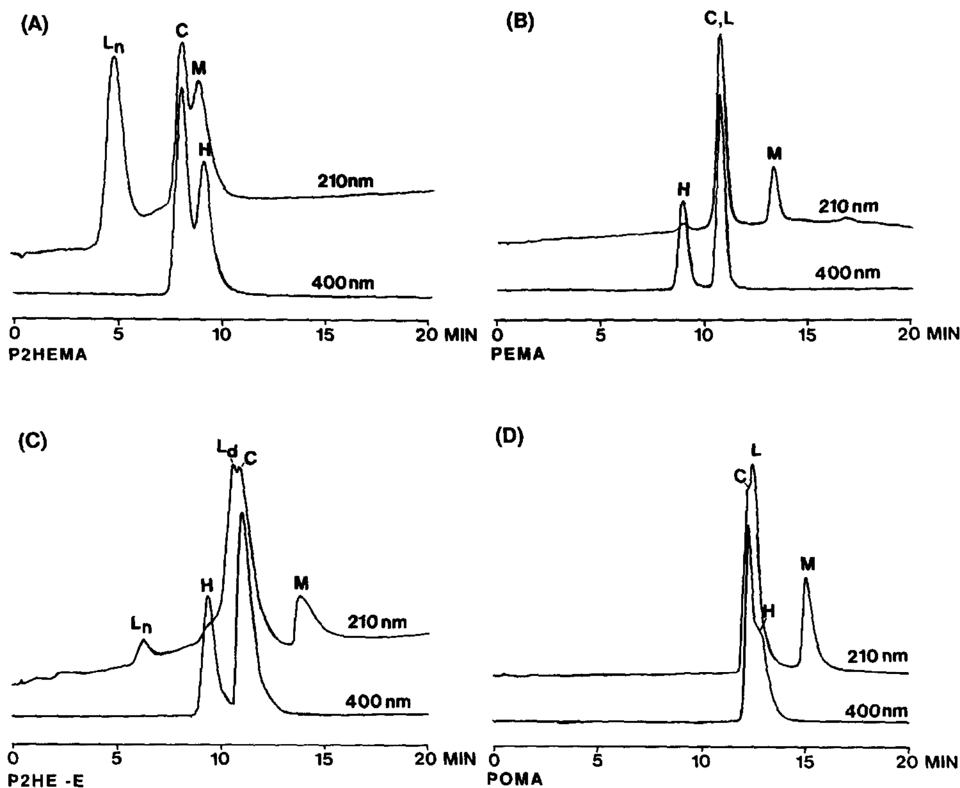


Fig. 4. Effect of hydrophobicity of porous LiChrospher packings Si 300 (10 μm) on RP-HPLC elution profiles of proteins. (A), (B), (C) and (D) show the elution profiles obtained on the P2HEMA, PEMA, P2HE-E and POMA packings, respectively. Mobile phase, linear A–B gradient (4% acetonitrile/min) at a flow-rate of 1 ml/min, where A is 0.05% aqueous TFA and B is 0.05% TFA in 80% aqueous acetonitrile, both A and B containing 0.1 *M* $NaClO_4$; temperature, 25°C. Ln, Ld, C, M and H denote native lysozyme, denatured lysozyme, cytochrome *c*, myoglobin and haeme, respectively. Sample mixture dissolved in water.

chlorate. In a similar manner to that observed for the Monospher packings (Fig. 2), there is a switch in elution order of the three proteins between the P2HEMA packing (Fig. 4A) (native lysozyme, followed by cytochrome *c* and, finally, myoglobin) and the POMA packing (Fig. 4D) (cytochrome *c*, followed by unolded lysozyme and, finally, myoglobin), although, under the run conditions employed, cytochrome *c* and denatured lysozyme are barely separated on the latter column. However, the P2HE-E and PEMA packings exhibit some interesting intermediate profiles. On the P2HE-E column, most of the lysozyme is now eluted in its unfolded form just prior to cytochrome *c*. With the PEMA packing, all of the lysozyme is now in its unfolded form, and is co-eluted entirely with cytochrome *c*. The latter observation is in contrast to the elution behaviour of lysozyme on the Monospher PEMA packing, where the protein is eluted in only a partially unfolded state (Fig. 2B). This apparent greater stability of lysozyme on the Monosper packing compared with the LiChrospher PEMA packing may be due to the inherent properties of the porous packing material (Fig. 4C) compared with the Monospher column (Fig. 2B). Apart from the changes in the chromatographic patterns of the three proteins on the four porous packings, there are also selectivity differences between the non-porous and porous versions of the same polymer coatings even allowing for the different run conditions employed on the porous and non-porous packings. This is especially clear from the elution behaviour of cytochrome *c* and unfolded lysozyme on the LiChrospher PEMA and POMA packings (Fig. 4)

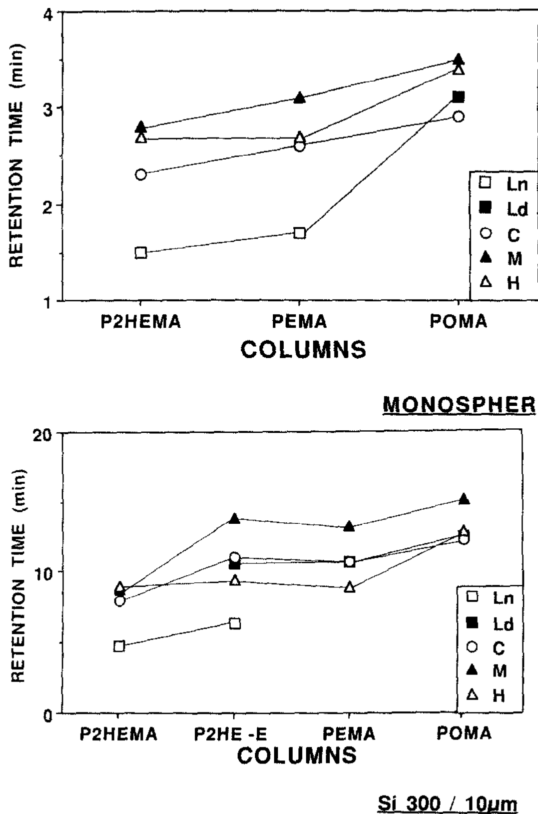


Fig. 5. Summary of stationary phase selectivities for RP-HPLC separation of lysozyme, cytochrome *c* and myoglobin. Mobile phase: Monospher packings, see Fig. 2; LiChrosper packings, see Fig. 4. □ = Ln; ■ = Ld; ○ = C; ▲ = M; and △ = H, denoting, native lysozyme, denatured lysozyme, cytochrome *c*, myoglobin and haeme, respectively.

compared with that observed on their Monospher counterparts (Fig. 2).

Fig. 5 summarizes the retention behaviour of the three proteins on the LiChrospher and Monospher packings. As might be expected, there is a general increase in protein retention times on both series of packings as the hydrophobicity of the polymer coating is increased from P2HEMA to PEMA to POMA. The most dramatic increase occurs with lysozyme as it is converted from its native (□) to its denatured (■) form.

Selective unfolding of proteins by temperature manipulation

Although the primary cause of protein unfolding in standard RPC is the hydrophobicity of a reversed-phase matrix, changes in temperature can also have a dramatic effect on protein stability [2,7,8,10,11]. Proteins are increasingly unfolded as the temperature is raised, the extent of thermally induced unfolding being dependent on the lability of the specific protein involved. Indeed, several researchers have noted that differences on protein lability to temperature changes could be used to improve resolution of proteins with similar retention times [2,7,8,10,11]. Thus, it was felt that temperature manipulation during RP-HPLC of proteins on a specific polymer-coated support, as opposed to their resolution on packings of varying hydrophobicity, may add another dimension to the utility of the stationary phases described in this study.

As an initial test of this approach to RP-HPLC of proteins, lysozyme was subjected to RP-HPLC on the LiChrospher P2HEMA packing at temperatures ranging from 25 to 40°C (Fig. 6A). The P2HEMA stationary phase was chosen as lysozyme had been shown to be in its native state on this packing at room temperature (25°C) (Figs. 3D and 4A). From Fig. 6, a progressive unfolding of lysozyme (native lysozyme, Ln, to unfolded lysozyme, Ld) is observed as the temperature is raised from 25 to 40°C. At 25°C, lysozyme is in a fully native state (Ln), unfolding to a fully unfolded state (Ld) at

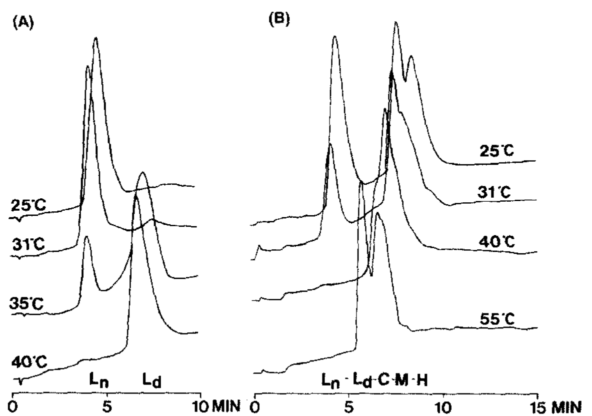


Fig. 6. Effect of temperature on RP-HPLC elution profiles of proteins on porous LiChrospher P2HEMA packing. (A) Lysozyme only; (B) mixture of lysozyme, cytochrome *c* and myoglobin. Mobile phase, linear A–B gradient (4% acetonitrile/min) at a flow-rate of 1 ml/min, where A is 0.05% aqueous TFA and B is 0.05% TFA in 80% aqueous acetonitrile; temperatures as indicated; absorbance at 210 nm. Samples dissolved in water.

40°C, as evidenced by the increase in protein retention time at the higher temperature. Intermediate temperatures of 31°C and, particularly, 35°C, produced chromatographic profiles indicating the presence of both native and unfolded lysozyme.

Fig. 6B shows the effect of increasing temperature on the resolution of the three-protein mixture (lysozyme, cytochrome *c* and myoglobin). Protein retention times in RP-HPLC generally decrease with increasing temperature, owing to increasing solubility of the solute in the mobile phase as the temperature rises [7,10,11,20,21]. In addition sharper protein peaks, often leading to improved resolution due to a more rapid transfer of the solutes between the stationary and mobile phases, generally accompanies a rise in temperature. It is apparent from Fig. 6B that there is indeed an overall sharpening of peptide peaks with an increase in temperature, together with a decrease in retention times of the two proteins (cytochrome *c* and myoglobin) already denatured at room temperature (25°C). However, the resolution of this particular peptide mixture is clearly not improved with increase in temperature, owing to the gradual appearance of unfolded lysozyme in an elution position close to the other two proteins. Wider band widths at room temperature are offset by the excellent separation of lysozyme from the other two proteins owing to the maintenance of its native state. Hence, although temperature manipulation during RP-HPLC of proteins may undoubtedly complement the selective protein unfolding properties of a stationary phase, this approach must be tailored to the specific protein mixture under investigation.

Application of polymethacrylate-coated silicas to preparative purification of proteins in their native conformation

The maintenance of the native protein during the preparative purification of proteins is frequently of concern to researchers, particularly where retention of biological acitivity is desired. The results of this study (Figs. 2–6) (reflecting similar observations of other researchers [7,10,11]) have demonstrated that the native state of proteins may be maintained during RP-HPLC through the employment of a mildly hydrophobic stationary phase and/or low temperatures, suggesting a potential preparative role for such packings. To test this potential further, polymethacrylate-coated silicas were applied to RP-HPLC of a multi-protein complex (stabilized by non-covalent interactions), rabbit skeletal troponin (RsTn), which was felt to be an even greater challenge than isolation of a single monomeric protein in its native conformation.

Isolation of RsTn and its individual protein components (TnT, TnI, TnC) had traditionally been carried out by classical open-column techniques, generally employing combinations of chromatography on ion-exchange resins [22–24]. However, as Cachia *et al.* [25] pointed out, these methods of purification are time consuming and result in the dispersion of the desired product(s) in large volumes of column effluent. The denaturing character of RP-HPLC has so far precluded advantage being taken of the powerful resolving capability of this technique for purifications of this kind.

Fig. 7 shows the elution profile of whole RsTn on Monospher P2HEMA (Fig. 7B), comparing it with the elution profiles of the three individual subunits run separately (Fig. 7A). Although maintenance of the three-protein complex is achieved at 5°C (also the temperature at which the individual subunits were chromatographed), as evidenced by the single peak, gradual dissociation of the subunits was evident as the temperature was raised.

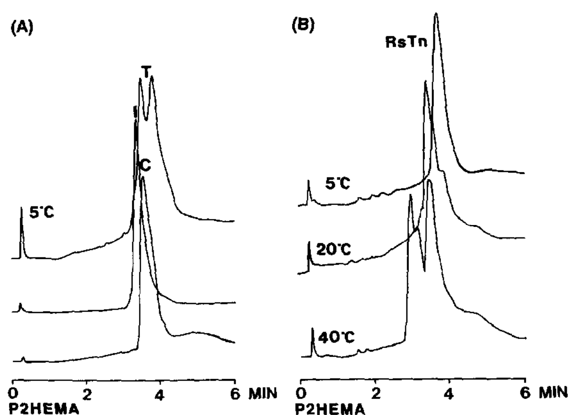


Fig. 7. RP-HPLC of rabbit skeletal whole troponin (RsTn) and individual subunits (TnI, TnT, TnC) on non-porous Monospher packings. (A) Subunits run individually on P2HEMA; (B) whole troponin run on P2HEMA. Mobile phase, linear A–B gradient (16% acetonitrile/min) at a flow-rate of 1 ml/min, where A is 0.05% aqueous TFA and B is 0.05% TFA in 80% aqueous acetonitrile, both A and B containing 0.1 *M* $NaClO_4$; temperatures as indicated; absorbance at 210 nm. Samples dissolved in water.

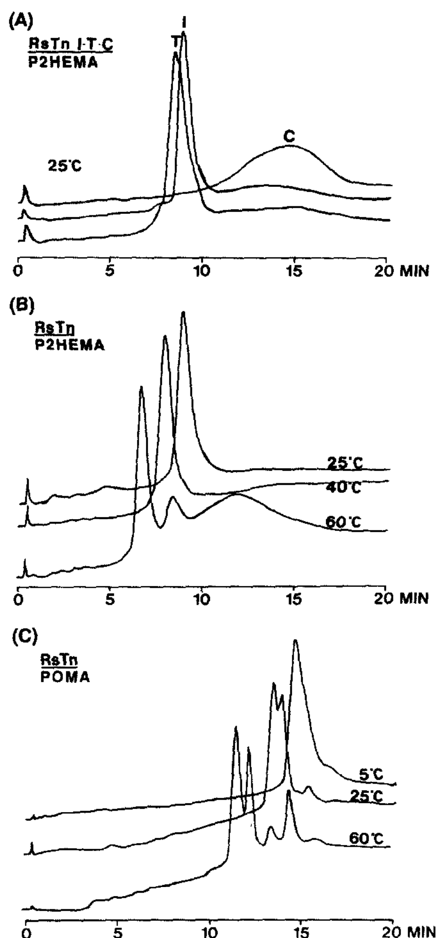


Fig. 8. RP-HPLC of rabbit skeletal whole troponin (RsTn) and individual subunits (TnI, TnT, TnC) on porous LiChrospher Si 300 (10 μm) packings. (A) Subunits run individually on P2HEMA; (B) whole troponin complex run on P2HEMA; (C) whole troponin complex run on POMA. Mobile phase, linear A–B gradient (4% acetonitrile/min) at a flow-rate of 1 ml/min, where A is 0.05% aqueous TFA and B is 0.05% TFA in 80% aqueous acetonitrile, both A and B containing 0.1 *M* $NaClO_4$; temperatures as indicated; absorbance at 210 nm. Samples dissolved in water.

The results in Fig. 8 suggest that the complex is even more stable on the LiChrospher P2HEMA packing, with a single peak being maintained even at 40°C (Fig. 8B). At first glance, this interesting observation is unexpected because, all other things being equal, non-porous materials are usually deemed to be less denaturing than porous materials, owing to an absence of pore effects. However, an explanation may lie with the original preparation of the packings. Thus, a minimum of *ca.* 1% (w/w) of polymer was employed to ensure a complete coating on the non-porous Monospher silica support (see Experimental). On the porous LiChrospher material, 10% (w/w) of polymer was employed (with concomitant loss of material during the prepolymer synthesis process), resulting in a lower value of % carbon/m^2 of support surface area compared with the Monospher material, *i.e.*, it is possible that, in this instance, the porous material is potentially less denaturing than the Monospher packings. The decrease in the retention time of the complex at 40°C compared with 25°C is consistent with previous observations of the effect of temperature on RP-HPLC elution profiles [7,10,11,20,21]. It is interesting that even on the LiChrospher POMA packing (Fig. 8C), containing the most hydrophobic polymer coating synthesized, it appears that the multi-protein complex may be maintained (if only barely, considering the broadness of the peak) at low temperature (5°C).

CONCLUSIONS

We have evaluated the potential of a novel concept for protein separations by reversed-phase chromatography on non-porous and porous polymethacrylate-coated silicas. Selective unfolding of proteins was achieved by varying the hydrophobicity of the polymer coat, permitting manipulation of the chromatographic pattern of analytical protein separations. In addition, it was also demonstrated that maintenance of the native state of a multi-protein complex, stabilized by non-covalent interactions, should be possible during preparative isolation. The results of this preliminary study, together with a companion paper which further characterizes these packings through their employment for separations of model peptides [26], suggests that the potential of these packings for polypeptide separations is very promising.

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