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PAPER

Monolithic porous layer open tubular (monoPLOT) columns for low pressure liquid chromatography of proteins†

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Glycidyl methacrylate–ethylene dimethacrylate (GMA-*co*-EDMA) based monolithic porous layer open tubular (monoPLOT) columns (0.05 mm I.D., monolithic layer thickness ≈ 5 μm) have been fabricated using an automated column scanning technique, providing UV polymerisation at 365 nm. Columns were chemically modified to obtain desired diol groups on the surface, and the longitudinal homogeneity of the stationary phase was profiled using scanning capacitively coupled contactless conductivity detector (sC^4D), before and after such modification. Using the automated scanning polymerisation technique, column-to-column production reproducibility, including longitudinal phase thickness, was within ±5% RSD. The prepared columns were tested to evaluate their liquid chromatographic stationary phase selectivity, efficiency and reproducibility, with a series of test protein mixtures. Under optimised gradient conditions, the separation of up to 8 proteins was demonstrated on the open tubular column (510 × 0.05 mm I.D.), with a column pressure drop of <1.5 MPa.

Introduction

Though the vast majority of routine separations in liquid chromatography (LC) are performed on standard-bore (~4.0 to 4.6 mm I.D.) columns, with wider bore columns used in preparative LC, a significant level of interest and development has recently been applied to miniaturised and micro-format LC systems (so-called 'micro-', 'capillary-' and 'nano-LC'). This has resulted in a considerable variety of new commercially available capillary columns for various chromatographic modes and applications. These developments have occurred for a number of reasons, although generally it is accepted that the use of the columns with reduced I.D., together with, or without a simultaneous reduction in stationary phase particle size, can potentially provide higher separation efficiency and speed.[1] Additionally, miniaturisation of the LC columns obviously means lower mobile phase consumption, less dilution of the sample in the column, which improves detection sensitivity (although not necessarily LODs), and facilitates the analysis of very small samples, which can be of great importance for forensic analysis, medicinal diagnostics *etc.* The capillary columns commonly encountered can be subdivided according to the type and physical format of the stationary phase. Particle packed[2] and monolithic columns[3,4] are the most common column formats in capillary-LC (capLC), with simple open tubular (OT)[5,6] and porous layer open tubular (PLOT)[7,8] columns finding less application in capLC, but remaining the dominant formats in capillary gas chromatography (GC).[9] Capillary electrophoretic methods, such as capillary zone electrophoresis (CZE), are also predominantly OT capillary based methods, with capillary electrochromatographic (CEC) methods having been developed using particle packed, monolithic and PLOT type columns.[10–15]

However, from as far back as the late 1970s there have been attempts to apply open tubular format columns to LC separations,[16] although in most cases practical and instrumental limitations have resulted in only limited success being reported. These limitations include the obvious issue of low stationary phase capacity and difficulty in producing a stable longitudinally homogenous adsorbed or surface bonded stationary phase, together with the lack of sensitive small volume detectors compatible with micro-bore capillary columns, and the lack of LC instrumentation to provide effective generation of mobile phase gradients at sub-μL min^{-1} flow rates. Although the instrumentation issues have now largely been resolved, with many gradient capLC systems available commercially, the preparation of open tubular columns for high efficiency separations still remains a challenge, particularly surface bonded porous phases of a uniform layer thickness, which can provide sufficient retention of analytes and sample loading capacity. One of the solutions to this problem can be the use of porous polymeric stationary phases. Firstly, this type of stationary phase can provide a highly developed surface area which is relatively physically and chemically stable (especially at extreme pH

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values). Secondly, the use of porous polymeric stationary phases provides the possibility of varying the surface chemistry, through simple surface modification procedures, which can be carried out *in situ*. Up to date there have been two strategies developed for the preparation of polymeric phase open tubular columns. One requires the pre-fabrication of polymer particles, which are then bonded to the inner wall of the capillary,[17] the second one is based on the *in situ* polymerisation of the immobilised polymeric phase as a single (monolithic) porous layer.[18,19]

Up to date there have been relatively few studies published on the application of OT and PLOT columns in LC, however, some impressive demonstrations of the potential of these columns for LC have been shown.[19–23] For example, Yue *et al.*[19] used a 4.2 m × 0.01 mm I.D. poly(styrene-divinylbenzene) (PS-DVB) PLOT column, combined with a 30 cm monolithic PS-DVB pre-column for solid phase extraction (SPE), for the efficient separation of peptides. Later the same research group[19–22] extended the application of the reversed-phase PS-DVB PLOT column (3.2 m × 0.01 mm I.D.), in combination with a traditional strong cation-exchange column (SCX), to the two-dimensional separation of peptides. The method required only a very small volume of protein digest sample, equal to only 500 ng, and permitted the identification of over 2700 peptides, associated with 850 unique proteins. Luo *et al.*[20] also produced a 2.5 m × 0.01 mm I.D. amino-group bonded poly(vinylbenzyl chloride-divinylbenzene) PLOT column, which was applied to the analysis of *N*-linked glycans, using a hydrophilic interaction chromatographic approach (HILIC). Rogeberg *et al.*[23] also recently successfully demonstrated the potential of PS-DVB PLOT columns for the efficient separation of intact proteins.

In the work presented herein, relatively short, 0.30 m × 0.05 mm I.D. monolithic porous layer open tubular (monoPLOT) columns were prepared by *in situ* surface layer co-polymerisation of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA), followed by further stationary phase chemical modification for chromatographic application. Significantly, the longitudinal homogeneity of the obtained polymeric stationary phase layer could be characterised non-destructively using scanning capacitively coupled contactless conductivity detector (sC^{4}D), both before and after any chemical modification steps. The fabricated monoPLOT columns were evaluated for the separation of test protein mixtures under low pressure conditions, using simple mobile phase gradients. The effects of gradient profile, mobile phase pH and composition, and column temperature were studied, with optimised conditions allowing the complete separation of the test protein mixture on the short monoPLOT column, with column pressure drops of below 2 MPa.

Experimental

Chemicals and reagents

Glycidyl methacrylate, ethylene dimethacrylate, cyclohexanol, 1-decanol, 4,4′-bis(*N*,*N*-dimethylamino)benzophenone, trimethoxysilylpropyl methacrylate, trifluoroacetic acid (TFA), sodium hydroxide, hydrochloric acid and sulfuric acid were all purchased from Sigma-Aldrich (Gillingham, UK). The thermal initiator 1,1′-azobisisobutyronitrile (AIBN) was obtained from DuPont (Le Grand, Sacconex, Switzerland). Solvents used for synthesis, washing the monoPLOT columns and for the preparation of mobile phases, namely acetonitrile (ACN), methanol, toluene and acetone were obtained from LabScan (Gliwice, Poland). Teflon-coated fused silica capillary, 0.05 mm I.D., 0.375 mm O.D., was purchased from Composite Metal Services Ltd. (Charlestown, UK). Deionised water was supplied from a Milli-Q system (Millipore, Bedford, USA).

Instrumentation

A Knauer Smartline 100 pump (Knauer GmbH, Berlin, Germany) was used to deliver reagents used for the flow-through modification of the monoPLOT columns. For column thermostatting during the modification procedure, a Mistral column oven (Spark-Holland, Emmen, Netherlands) was used. For the evaluation of column longitudinal homogeneity, both before and after modification, a TraceDec capacitively coupled contactless conductivity detector (C^{4}D) (Innovative Sensor Technology GmbH, Strasshof, Austria) was used. Settings for scanning the column before and after the treatment: frequency, 3 × HIGH; voltage, −6 dB; gain, 50% and offset, 0. For the data acquisition TraceDec Monitor V. 0.07a software (Innovative Sensor Technology GmbH, Strasshof, Austria) was used. A SputterCoater S150B (BOC Edwards, Sussex, UK) was utilised for coating the monoPLOT sample with a 60 nm gold layer prior to scanning electron microscopy (SEM) characterisation, which was performed on an S-3400N instrument (Hitachi, Maidenhead, UK). For the chromatographic study, an Ultimate 3000 nano-HPLC system was used, incorporating a HPG-3x00RS binary pump, WPS-3000RS autosampler, TCC-3000 RS thermostatted column compartment and an MWD-3000RS multiple wavelength detector, with a 3 nL detector cell (Dionex, Sunnyvale, CA, USA). For data acquisition Chromeleon 6.8 software (Dionex, Sunnyvale, CA, USA) was utilised.

Preparation of monoPLOT columns

Fused silica capillaries were initially pre-treated through activation of the surface silanol groups using sequential flushing with 1 M NaOH, deionised water, 0.1 M HCl, deionised water, and acetone. The pre-treated capillary was silanised using a 50 wt% solution of trimethoxysilylpropyl methacrylate in toluene at 60 °C for 24 h. The open tubular GMA-*co*-EDMA monolith was produced utilising UV initiated polymerisation at 365 nm using a novel automated capillary "UV light-scanning" technique (patent pending). The GMA-*co*-EDMA open tubular monolith was formed using a monomer mixture consisting of 24 wt% glycidyl methacrylate, 16 wt% ethylene dimethacrylate, 30 wt% cyclohexanol, 30 wt% 1-decanol and 1 wt% 4,4′-bis(*N*,*N*-dimethylamino)benzophenone (in respect to monomers). Silanised capillaries (300 mm long) were completely filled with the polymerisation mixture and repeatedly exposed to light from the scanning source (365 nm), along the length of the capillary column at a uniform scan rate, for an optimised length of time (up to 25 min). The monolithic phase is formed during this perpendicular illumination. Polymer growth is propagated from the modified capillary wall inwards, evenly from the entire inner capillary surface, resulting from what is likely to be an evanescent

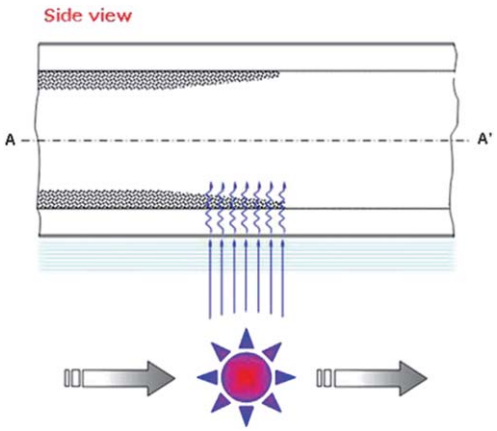


Fig. 1 Schematic diagram of the automated scanning photo-polymerisation technique developed for the preparation of open tubular monolithic columns.

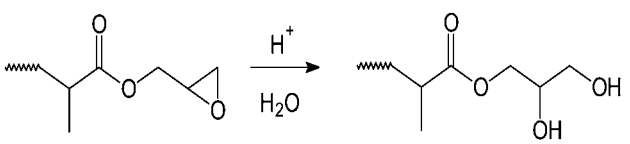
wave effect along the internal boundaries during capillary illumination (Teflon/fused silica, fused silica/polymerisation mixture). As the monolith forms inwardly, exposure time can be used to control monolith depth, in this case to ~5 μm. A schematic of which is shown in Fig. 1.

Following polymerisation, residual monomers and solvents were washed off the monoPLOT column with methanol at a flow rate of 5 μL min^{-1}, and the column dried under a flow of nitrogen for 15–20 min. Scanning electron microscopy (SEM) was used to characterise the open tubular monolith at multiple positions along the length of the capillaries to confirm structural homogeneity and measure monolithic thickness to the capillary wall. All samples were gold-sputtered with a 60 nm layer prior to imaging in order to minimise charging and improve the image contrast.

For evaluation and comparison of column permeability, a fully monolithic (non-PLOT) GMA-*co*-EDMA capillary column (270 × 0.05 mm I.D.) was prepared. Here, the fused silica capillary was pre-treated and silanised as described above, and the monomer mixture used consisted of 24 wt% glycidyl methacrylate, 16 wt% ethylene dimethacrylate, 30 wt% cyclohexanol, 30 wt% 1-decanol and 1 wt% AIBN (in respect to monomers). Polymerisation was performed for 20 hours at 60 °C. After completion of polymerisation, the prepared monolith was washed with ACN overnight at a flow rate of 1 μL min^{-1}.

Modification of monoPLOT column

A simple modification procedure was utilised to convert surface epoxy groups to polar diol groups, according to reaction Scheme 1. Modification simply involved column flushing for 8 h with 1% aqueous solution of H_2SO_4 at 60 °C.



Scheme 1 Modification of surface epoxy groups to polar diol groups using dilute sulfuric acid solution at 60 °C.

Results and discussion

Column characterisation

To characterise the prepared monoPLOT column two approaches were used. One of the methods was SEM imaging, which was used to confirm the column morphology and evaluate the thickness of the porous layer. This method involved the sacrifice of a series of prepared monoPLOT columns during optimisation of the scanning polymerisation technique, which were cut in 1 cm pieces along the complete column length and SEM images of each cross-section obtained (Fig. 2 shows a typical cross-sectional image). All images obtained showed that the porous layers formed within the capillaries were uniform in thickness and polymeric density, with an even layer of porous stationary phase of a thickness of approximately 5 μm. Fig. 3 shows an expanded SEM image confirming the integrity of the wall bonding, in this case achieved on a 100 μm I.D. fused silica capillary. The image clearly shows the typical globular porous structure of the GMA-*co*-EDMA monolith and lack of voids along the monolith–wall interface.

Although the use of SEM provides an accurate means to measure the thickness of the layer at each specified location, it is not a suitable method for the study of complete stationary phase homogeneity, as it is obviously destructive and rather time consuming. However, sC^4D has been previously demonstrated as an effective non-invasive technique for this very purpose.[24–27] In this application, sC^4D can also be utilised for comparison of replicate columns and evaluation of the surface modification procedure. The overall sC^4D response for the capillary-housed

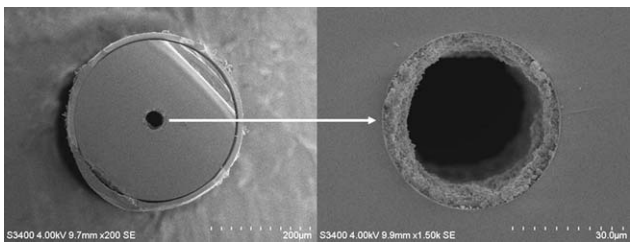


Fig. 2 Typical cross-sectional SEM images of the GMA-*co*-EDMA monolithic porous layer open tubular column (50 μm I.D.).

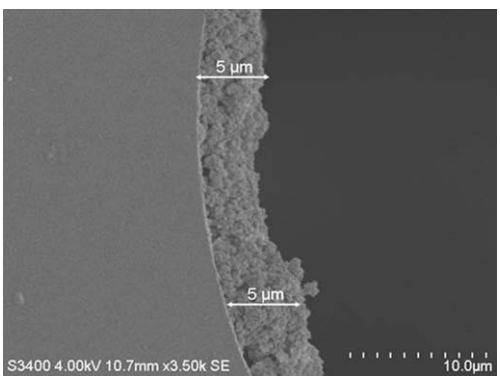


Fig. 3 SEM image of the GMA-*co*-EDMA monolith-capillary wall interface, for a 100 μm I.D., monolithic porous layer open tubular column.

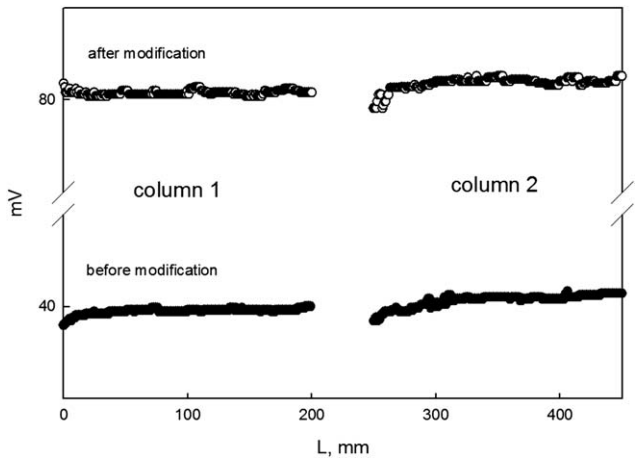


Fig. 4 Scanning C^4D of two individually prepared 50 μm I.D. monoPLOT columns, before and after modification to provide surface diol groups.

monoPLOT columns developed here is the sum of signal contributions originating from the residual silanol groups on the surface of the silica housing, from the monolith layer itself and attached functional groups on the surface of the polymer, and of the solution phase within the capillary. For the unmodified monoPLOT phase, any significant longitudinal variation observed is then a combination of variation in the capillary housing itself (assumed to be negligible), and from the thickness and density of the monolithic phase, which indirectly effects the volume of solution phase within the detection zone, and is reflected within the detector response. As here the monolithic phase itself was converted from a non-polar to polar phase, a low conducting solution phase, in this case simply dionised water, allowed a single profile of the columns before and after modification to confirm both physical and chemical homogeneity of the porous layer phase (see Fig. 4). Hence, the presence or absence of physical gaps in the porous layer, or significant thickness changes, or variation in the concentration of charged groups on the surface of the stationary phase, could be confirmed using this simple technique.

Two individually prepared 270 mm long GMA-*co*-EDMA monoPLOT columns were scanned, recording the signal every 1 mm (Fig. 4, bottom trace). After conversion of the two columns to a surface diol functionality, the procedure was repeated (Fig. 4, top trace). The recorded profiles confirmed that the stationary phase layer exhibited a homogeneous structure, as the profiles for non-modified columns were practically flat without significant deviations, and the same pattern was observed after modification. Also, significantly, it can be seen from comparing the scans of the two columns that column-to-column fabrication reproducibility, including surface modification, was excellent, with less than ±5% variation between the two replicate columns, both before and after modification.

Chromatographic evaluation

Backpressure and flow rate. Karger and co-workers[19–22] used Darcy's law,[28] to calculate the permeability of long (4.2 m × 0.01 mm I.D.) PLOT columns, using $k = \upsilon(\mu\Delta x/\Delta P)$ (where k is permeability, υ is linear velocity, μ is solution viscosity, Δx is column length and ΔP is pressure drop). This approach provided backpressures of ~20 MPa at flow rates of 20 nL min^{-1}. It was shown that the permeability value for the PLOT column was 1.3 × 10^{-12} m^2, which was 4 times lower than for the equivalent bare capillary of 0.01 mm I.D.

The same approach was applied to the monoPLOT columns produced within this study. The 270 × 0.05 mm I.D. monoPLOT column provided a backpressure of 1.2 MPa at a flow rate of 0.2 μL min^{-1} using water as the mobile phase, and the calculated permeability found to be 7.2 × 10^{-12} m^2, while for the bare 0.05 mm I.D. fused silica capillary of the same length this value was found to be approximately 4.5 times higher. The lower permeability and higher pressure drop for the monoPLOT column are certainly due to the presence of porous layer, which reduces the internal diameter of the open tube. Compared to the 0.01 mm I.D. PLOT column,[19] the permeability of the PLOT column studied in the current work was 5.5 times higher, which was obviously due to the larger diameter of the open tube.

The permeability of the monoPLOT column was also compared to that of a fully monolithic GMA-*co*-EDMA capillary column (non-PLOT), prepared in a 270 × 0.05 mm I.D. fused silica capillary. For this column the backpressure was 6 MPa at a flow rate of 1.3 μL min^{-1} for ACN. The calculated permeability was found to be 1.9 × 10^{-14} m^2, which is 360 times lower than for the monoPLOT column.

In the work of Karger and co-workers,[19–22] and later by Rogeberg *et al.*,[23] PS-DVB PLOT columns based upon 10 μm I.D. fused silica capillaries, of up to 5 m in length, with approximately 0.5 to 1 μm thick porous layers were investigated. The long PLOT columns were used in gradient LC applications, so no data on column efficiency for intact proteins or digest peptides were presented from either group. However, under such conditions, despite the long analysis times, peak capacities were impressive for open tubular LC.

In the work presented herein, a similar ratio of capillary I.D. to porous layer thickness was achieved (10 : 1), although the resultant shorter wider bore columns exhibited a much lower column backpressure. However, a 10-fold reduction in capillary column length compared to the above studies would significantly reduce peak capacities for such separations, although very low pressure separations of simpler mixtures of large biomolecules and intact proteins were achieved.

Initial chromatographic performance was investigated using a test mixture of 8 proteins, namely ribonuclease, insulin, cytochrome C, horseradish peroxidase, carbonic anhydrase, enolase, alcohol dehydrogenase and phosphorylase. A simple 20 min ACN–water mobile phase gradient (constant 0.1% TFA) was applied from 0 to 90% ACN, with a column temperature of 20 °C to evaluate relative retention of each of the above analytes on the monoPLOT column. The column backpressure over the flow rate range of 0.1 to 1.5 μL min^{-1} was also recorded under both starting (100% water) and final gradient conditions (90% ACN). The flow rate was increased at increments of 100 nL min^{-1} and the backpressure was allowed to stabilise at each flow rate value. Fig. 5(a) shows that backpressure linearly increases with the increase in flow rate under both conditions, indicating a stable porous layer. The highest flow rate applied was 1.5 μL min^{-1}, giving the column backpressure of only 6 MPa (excluding extra-column system backpressure).

The above mobile phase gradient was applied to the separation of the test protein mixture at each of the above flow rates. Fig. 5(b) shows the initial overlayed chromatograms of the resultant separations for each of the 8 proteins.

Temperature effects. The effect of capillary column temperature on the separation of the protein test mixture was also studied at 10, 20, 30, 40, 50 and 60 °C. An increase of temperature can result in the increase of column efficiency due to an improvement in mass-transfer, but at the same time elevated temperatures can potentially affect the structure of proteins, and as a result, their retention. Overall, an increase in column temperature caused a reduction in retention for all proteins. Some minor changes in selectivity were observed over this temperature range, and it was observed that for this particular set of proteins, a temperature of 20 °C showed the best overall resolution. However, no dramatic improvements in peak efficiencies were noted under these conditions. It should also be noted that the peak asymmetry increased with the increase of temperature. Thus, for cytochrome C the peak asymmetry increased from 1.05 to 1.75.

The relationship between the temperature and gradient retention factor (k_g) is illustrated in the Van't Hoff plot shown in Fig. 6 constructed using retention data from the repeat injection of the mixed protein standard at increasing temperature. Gradient retention factor k_g can be determined as:[29]

$$k_g = (t_r/t_m) - 1 \quad (1)$$

where t_r is the retention time of the compound of interest and t_m is the retention time of an unretained compound or hold-up time. Fig. 6 shows not only a decrease in the retention of proteins of interest, but also a maximum of selectivity at 20 °C. The change in the slope of ln k_g at 20 °C for carbonic anhydrase and alcohol dehydrogenase and at 50 °C for ribonuclease and insulin may be related to the change of protein conformation at these temperatures.

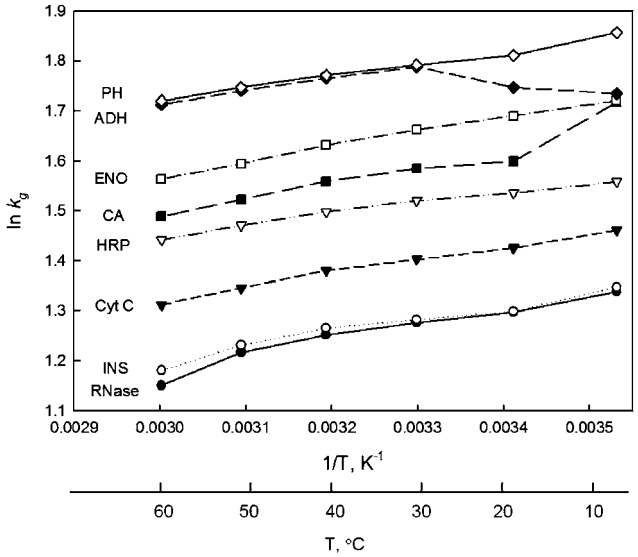


Fig. 6 Van't Hoff plot for the separation of proteins on a 27 cm monoPLOT column. Chromatographic conditions as in Fig. 4.

Column capacity. Column capacity and injection volume for short open tubular columns is obviously an important parameter in LC separations. To investigate the loading capacity of the monoPLOT columns, increasing sample volumes of a 0.1 mg

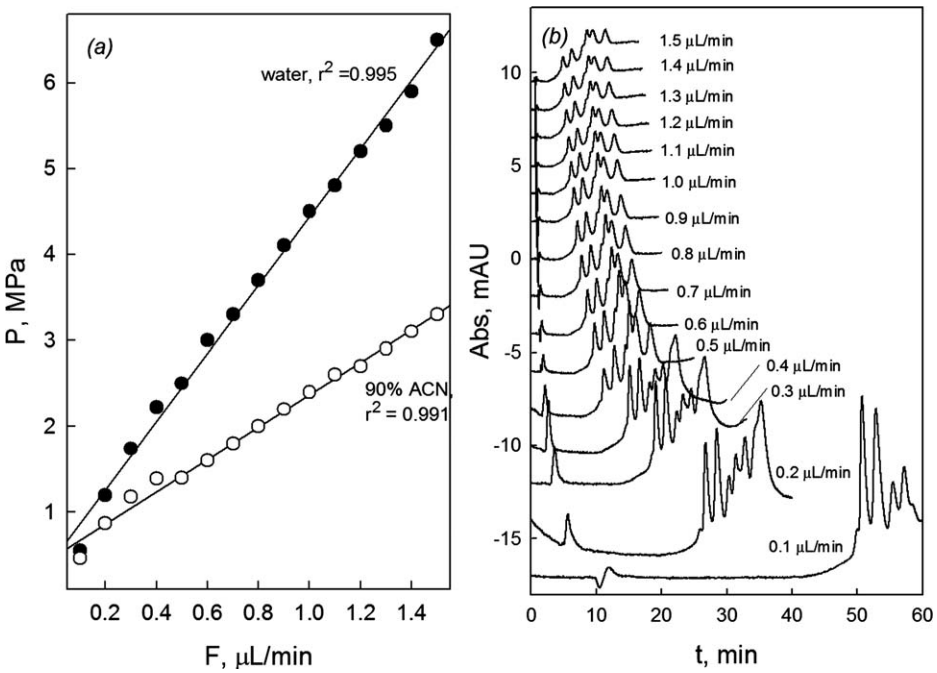


Fig. 5 (a) Column backpressure dependence on mobile phase flow rate. (b) Overlayed chromatograms of 8 proteins on the monoPLOT column. Mobile phase A: 0% ACN, 0.1% TFA; B: 90% ACN, 0.1% TFA. Gradient elution: 0.0–20.0 min from 100% A to 100% B; 20.0–20.1 min from 100% B to 100% A; 20.1–60 min 100% A. Column: diol functionalised GMA-*co*-EDMA monoPLOT, 270 × 0.05 mm I.D. UV detection at 214 nm. Temp. = 25 °C.

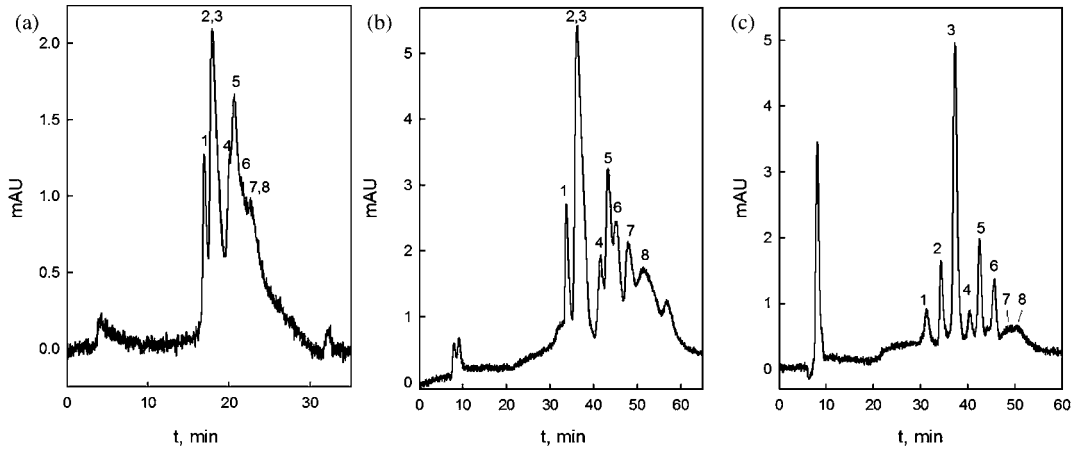


Fig. 7 The effect of mobile phase pH and concentration of TFA on the separation of proteins: 1—ribonuclease, 2—insulin, 3—cytochrome C, 4—horseradish peroxidase, 5—carbonic anhydrase, 6—enolase, 7—alcohol dehydrogenase and 8—phosphorylase. Column: diol functionalised monoPLOT (510 × 0.05 mm I.D.) Gradient conditions as in Fig. 4. TFA concentrations (a) 0.01%, pH 3.4, (b) 0.05%, pH 2.8, (c) 0.2%, pH 1.9. Flow rate = 0.4 μL min^{-1}. UV detection at 210 nm (blank baseline subtracted).

mL^{-1} cytochrome C standard were injected on a 270 × 0.05 mm I.D. column, with gradient elution from 0 to 90% ACN, 0.1% TFA. Injection volumes were varied from 10 nL to 0.5 μL. The results from these increased volume injections showed that peak retention remained constant over the injection volume range studied, although the asymmetry of the cytochrome C peak increased with the increase of injection volume from 10 nL to 100 nL. Above 100 nL, up to 0.5 μL, no significant further change was observed. From this investigation, an optimal injection volume, resulting in a peak asymmetry value close to 1, was found to be 50 nL, which is equal to 10% of column volume. Peak asymmetry data plotted against sample injection volume can be seen as ESI Fig. S1†.

The effect of TFA on the separation of proteins. Finally, the effect of TFA concentration in the mobile phase on the separation of proteins was studied. The concentration of the acid in the mobile phase is clearly expected to affect the structure and charge of the protein, and hence the separation selectivity on the monoPLOT phase. The separation of ribonuclease, insulin, cytochrome C, horseradish peroxidase, carbonic anhydrase, enolase, alcohol dehydrogenase and phosphorylase was investigated at different TFA concentrations: 0.01, 0.05, 0.1, 0.15 and 0.2% in the mobile phase. Fig. 7 shows several chromatograms obtained using (a) 0.01% TFA (pH 3.4), (b) 0.05% TFA (pH 2.8) and (c) 0.2% TFA (pH 1.9). (The chromatograms shown were achieved using two coupled monoPLOT columns, to give an overall effective column length of 510 mm.) Chromatograms resulting from other conditions investigated can be viewed in the ESI Fig. S2†. It can be seen that the peak shape and resolution improved with the increase of acid concentration. There was also a noticeable increase in retention of the proteins from 0.01% to >0.05% TFA. Although, under the conditions shown for Fig. 7(c), run times were relatively long, the chromatography achieved is indeed relatively good for such monoPLOT columns of these dimensions, operating under such very low pressure conditions. However, it is clear that further optimisation of flow rate could enable a significant reduction in overall run time if required, and that further surface chemistries could be investigated to increase the range of potential applications. >From the test mixture analysed in this study, the baseline resolution of proteins 1–7 could be achieved, with only proteins 7–8 showing co-elution.

Conclusions

The results presented herein have demonstrated the potential low pressure liquid chromatographic applications of novel short (270 and 510 mm) monoPLOT columns. Although their application here was to the separation of relatively simple test mixtures of proteins, further investigation of more selective surface chemistries is underway for both targeted low pressure separation and extraction applications.

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