Journal of Chromatography, 586 (1991) 21–26
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 547

Continuous beds for standard and micro high-performance liquid chromatography

Jia-Li Liao, Rong Zhang and Stellan Hjertén*

Department of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala (Sweden)

(First received April 24th, 1991; revised manuscript received May 27th, 1991)

ABSTRACT

Conventional high-performance liquid chromatographic columns are built up of small, uniform beads. The preparation of the columns involves many expensive and cumbersome steps. This paper describes a simpler method, which is not based on the use of preformed beads. The chromatographic bed consists of a compressed gel plug with intercommunicating "channels", the "walls" of which are impermeable to proteins. The gel bed is formed in one step: a monomer solution, including ligands, is polymerized in the chromatographic tube under such conditions that the polymer chains aggregate into bundles, for instance, by hydrophobic interaction [the voids ("channels") thus created between the polymer bundles, are large enough to permit passage of eluent]. The gel plug is then compressed 10–15 fold, which decreases the average diameter of the "channels". The gel bed formed shows a resolution that, at constant gradient volume, is independent of or increases with an increase in flow-rate, a property that it shares with a compressed, non-porous agarose bed. The potential of a continuous gel bed has been previously illustrated by a cation-exchange chromatography experiment. This paper gives details of the preparation of this cation-exchanger, as well as of beds for anion-exchange and hydrophobic-interaction chromatography. The usefulness of the beds is demonstrated by the separation of model proteins on columns with the diameters of 6 and 0.3 mm. For the micro column both on- and off-tube detection were used. In the latter procedure the protein zones were transferred by a buffer flow to a conventional UV detector as they leave the gel bed, and then to a fraction collector. This detection technique has the advantage that any detector and any flow-cell for high-performance liquid chromatography can be used. The sample becomes diluted, but the resolution and the sensitivity are about the same as those obtained with standard micro-flow-cells (the same technique has been used successfully for micro-preparative high-performance capillary electrophoresis). A new method of preparing salt gradients for micro columns is presented.

INTRODUCTION

The preparation of a chromatographic bed involves many steps: the preparation of the beads; washing to remove the suspension medium; sieving or elutriation of the beads to a uniform size (if they are not made monodisperse in the first step); derivatization; packing the column; and testing for bed homogeneity by measurement of plate numbers. This procedure is time-consuming and expensive.

We have recently described a considerably simplified method, which also has the potential to give a more homogeneous bed than do conventional packing procedures and, consequently, higher resolution [1]. The new method is based on polymerization of the monomers (including ligands) directly in the chromatographic tube under such conditions that "channels" are created in the polymer plug formed. Following compression of the bed it is ready for use. In a note on this new technique we described a separation of proteins on a cation-exchanger [1]. The present paper deals with an extension of this earlier study to anion-exchange and hydrophobic-interaction chromatography (HIC), including experiments on micro-columns with on- and off-line detection.

EXPERIMENTAL

Materials and apparatus

Electrophoresis-purity reagents [N,N'-methylenebisacrylamide (BIS), ammonium persulphate,

0021-9673/91/$03.50

N,N,N′,N′-tetramethylethylenediamine (TEMED)] and ammonium sulphate (HPLC grade) were obtained from Bio-Rad Labs (Richmond, CA, USA); N-allyldimethylamine from Fluka (Buchs, Switzerland); acrylic acid from Merck (Schuchardt, Germany); and butyl acrylate from Aldrich (Steinheim, Germany).

The HPLC pump (Model 2150), the LC controller (Model 2152), and the recorder (Model 2210) were from Pharmacia LKB Biotechnology (Bromma, Sweden); and the UV monitor (Model 1306) from Bio-Rad-Labs. The syringe pump for off-tube detection of proteins separated on the micro HIC column (0.3 mm I.D.) was constructed by Mr. Per-Axel Lidström and Mr. Hans Pettersson of this institute. The stainless-steel tees were from Upchurch Scientific (Oak Harbor, WA, USA) and the PTFE tubing (0.3 mm I.D.) from Alltech (Deerfield, IL, USA).

The column tubes with 6 mm I.D. were made of Plexiglas [2]. A fused-silica tubing with 0.3 mm I.D. was purchased from SGE (Ringwood, Australia) and employed as the micro column tube. The same company supplied the 0.15-mm fused-silica tubing attached to the micro-column for on-tube detection of proteins in the eluate, and a 0.075-mm fused-silica tubing for the introduction of buffer gradient and sample. The design of the micro-column set-up is outlined in Fig. 1. The devices for on- and off-tube detection were the same as those we use routinely for high-performance capillary electrophoresis (see refs. 3 and 4, respectively).

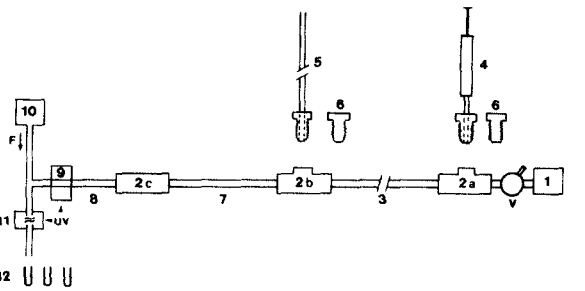

Fig. 1. Block diagram of the micro-column set-up. 1 = HPLC pump; 2a and 2b = zero dead-volume stainless-steel tees; 2c = union (ref. 5); 3 = gradient PTFE tubing (500 × 0.3 mm I.D.); 4 = 50-μl syringe; 5 = fused-silica tubing (150 × 0.075 mm I.D., volume 0.64 μl) for the introduction of buffer gradient and sample; 6 = stainless-steel nut (without bore); 7 = micro-column (fused-silica tubing, 70 × 0.3 mm I.D., containing the compressed bed); 8 = post column (fused-silica tubing, 30 × 0.15 mm I.D.) for on-line detection; 9 = on-tube detector; 10 = syringe pump; 11 = off-tube detector; 12 = fraction collector; v = valve.

Column preparation

Preparation of continuous beds for anion- and cation-exchange chromatography. N,N′-Methylenebisacrylamide (0.24 g) and N-allyldimethylamine (0.12 ml) were dissolved, with stirring, in 9.5 ml of 0.01 *M* sodium phosphate (pH 6.4). The pH was adjusted to 7 with 2 *M* HCl (*ca.* 400 μl). Ammonium sulphate (0.5 g) and 100 μl of a 10% (w/v) aqueous solution of ammonium persulphate were added. Following deaeration and addition of 100 μl of the catalyst [a 5% (v/v) solution of TEMED] the reaction mixture was poured into a 350 × 6 mm I.D. column tube [the final concentration of N,N′-methylenebisacrylamide was thus *ca.* 2.4% (w/v)]. After polymerization for 5 h, 0.01 *M* Tris–HCl (pH 8.5) was pumped into the column tube at a flow-rate of 0.5 ml/min. During this step the height of the gel plug decreased continuously. The pumping was not interrupted until the height became constant (*ca* 60 mm). The gel plug was then manually compressed further with the aid of the upper piston. The final height of the gel plug was 35 mm. The piston was fixed at this position to prevent the gel plug from expanding on elution at lower flow-rates. The compression thus decreased the height of the gel plug 10-fold.

We have previously shown the usefulness of continuous beds for the separation of proteins on a cation exchanger [1]. The method for the preparation of the bed was similar to that described above. The details were as follows. With stirring, 0.24 g of N,N′-methylenebisacrylamide was dissolved in 10 ml of 0.05 *M* sodium phosphate (pH 6.8), which was then supplemented with acrylic acid (0.005 ml), ammonium sulphate (0.45 g) and 0.1 ml of a 10% (w/v) aqueous solution of ammonium persulphate. The polymerization was initiated by adding 0.1 ml of a 5% (w/v) aqueous solution of TEMED to the deaerated solution. This monomer solution was poured into the column tube. Following polymerization, the bed was compressed as described for the anion exchanger.

Preparation of a continuous bed for hydrophobic-interaction chromatography. With stirring, 0.48 g of

N,N'-methylenebisacrylamide was dissolved in 20 ml of water. Following addition of 0.08 ml of butylacrylate, 0.3 g of ammonium sulphate and 200 μl of a 10% (w/v) solution of ammonium persulphate, the mixture was deaerated and supplemented with 200 μl of a 5% (w/v) solution of TEMED. Both the 450 × 6 mm I.D. tube and the 600 × 0.3 mm I.D. tube were filled with this catalysed monomer solution, which was allowed to polymerize for 5 h.

The 6-mm diameter bed was compressed to a height of 38 mm by pumping with a 0.01 *M* sodium phosphate solution (pH 7.0) containing 2.5 *M* ammonium sulphate, followed by manual pressing down of the piston as described above for the anion-exchange column.

The micro-column bed was prepared as follows. A union (2c in Fig. 1) containing a metal frit (pore diameter 2 μm) was attached to the fused-silica tubing containing the HIC bed (0.3 mm I.D.) [5]. With the aid of the HPLC pump 1, the bed was compressed at a flow-rate of 0.01 ml/min from a height of 600 mm to 120 mm, and then further to 70 mm by increasing the pressure to 100 bar for 5 min. At this stage the nuts 6 were screwed into the tees 2a and 2b. The column tubing above the compressed gel bed was cut off. The lower segment containing the gel was coupled to the PTFE tubing 3 via the tee 2b.

The formation of small-volume salt gradients for elution of the microcolumn

A 8.5-ml linear salt gradient was generated in a 10-ml cylinder (13 mm I.D.) with the aid of the HPLC pump. The gradient was formed from 2.25 *M* ammonium sulphate in 0.01 *M* sodium phosphate buffer (pH 7.0) (at the bottom) to 0.25 *M* ammonium sulphate in the same buffer (at the top). Using a marking pen, the cylinder was graduated from the bottom into 17 equal sections. The distance between two divisions thus corresponded to 0.5 ml. After equilibration of the column bed by the pump 1 with 0.01 *M* sodium phosphate (pH 7.0) containing 2.25 *M* ammonium sulphate, the stainless-steel nuts (6 in Fig. 1) in the tees 2a and 2b were replaced by a 50-μl syringe 4 and a 0.6-μl tubing 5. Sodium phosphate (0.01 *M*, pH 7.0) containing 0.25 *M* ammonium sulphate was pressed into the gradient tubing 3 and the tubing 5 with the aid of the HPLC pump 1. The valve v was then closed. The free end of the tubing 5 was immersed into the gradient, and 2 μl of the solution at the centre of each section were taken up with the 50-μl syringe 4 (starting with the top section). The final volume of the gradient in tubing 3 was thus $2 \times 17 = 34$ μl (the volume of tubing 5 was only 0.64 μl).

The free end of the tubing 5, now filled with 0.01 *M* sodium phosphate (pH 7.0) containing 2.25 *M* ammonium sulphate, was dipped into the sample solution, and 1 μl of the sample was taken up into the tubing with the aid of the syringe 4. The tubing 5 was then immersed into the equilibration buffer, and 2 μl were taken up with the syringe 4 (the sample was in this way introduced into the gradient tubing 3). The syringe 4 and tubing 5 (with connecting nuts) were replaced by the nuts 6. Valve v was opened and pump 1 turned on. Since the minimum flow-rate of the pump was 0.01 ml/min, the connection between the pump 1 and the tee 2a was loosened until the flow-rate in the column 7 was 0.001 ml/min.

The pore size in the "walls" of the "channels" in the gel bed

The porosity was studied by molecular-sieve chromatography on the beds for both HIC and anion-exchange chromatography (AEC) with standard proteins of different molecular weights dextran 2000 (M $2 \cdot 10^6$) and sucrose as described for compressed beds of nonporous agarose beads [6,7]. A plot of elution volume against molecular weight showed that the proteins and the dextran did not penetrate the channel walls (sucrose probably penetrates only a thin surface layer, to judge from the observation in Fig. 2 that its elution volume differs only slightly from that of macromolecules).

Scanning electron microscopy of the continuous polymer beds

Compressed continuous polymer beds for HIC, AEC and cation-exchange chromatography (CEC) were removed from the column tubes and frozen immediately. Following freeze-drying, aliquots of the beds were examined by scanning electron microscopy. A micrograph of the HIC bed is shown in Fig. 3 (similar pictures were obtained for the AEC and CEC columns).

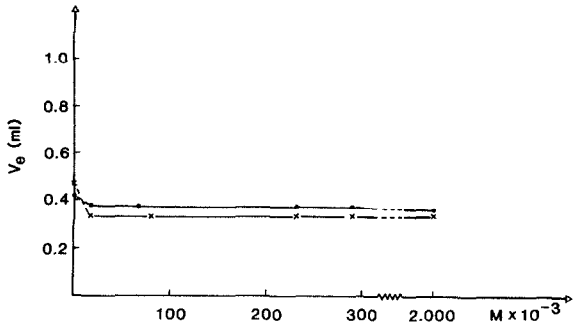

Fig. 2. Plot of elution volume (V_e) against the molecular weight (M) of some standard solutes chromatographed on a continuous bed. The figure illustrates that the "walls" of the "channels" in the continuous bed are impermeable to proteins. (●) AEC column (total volume 1.08 ml); (×) HIC column (total volume 1.11 ml).

The influence of the flow-rate on the resolution of the AEC column at constant gradient volume

The bed (35 × 6 mm I.D.) was equilibrated with 0.01 *M* Tris–HCl (pH 8.5). The sample consisted of 20 μg of each of the proteins myoglobin (M), haemoglobin (H), ovalbumin (O), bovine serum albumin (A) and *R*-phycoerythrin (P), dissolved in 40 μl of the equilibration buffer. The desorption was accomplished at the flow-rates 0.12, 0.25 and 0.50 ml/min by a 5.0-ml linear salt gradient generated from the equilibration buffer and the same buffer supplemented with 0.43 *M* sodium acetate (pH 8.5). ty similar experiments were performed during 3 months on the same column without any change in the appearance of the chromatograms, indicating a good reproducibility and stability of the column. The last run during this test period is presented in Fig. 4.

A comparison between on-tube and off-tube detection of proteins separated on the micro-column

The sample consisted of *ca.* 0.4 μg of each of the following proteins dissolved in 1 μl of the equilibration buffer: myoglobin (M), ribonuclease (R), ovalbumin (O), α-chymotrypsinogen A (C) and *R*-phycoerythrin (P). The separation of the proteins was accomplished with a 35-μl negative, linear gradient formed from 2.25 *M* ammonium sulphate in 0.01 *M* sodium phosphate (pH 7.0) (the equilibration buffer), and 0.25 *M* ammonium sulphate in the same phosphate buffer. The flow-rate was 1 μl/min.

For on-tube monitoring, the "flow cuvette" of the

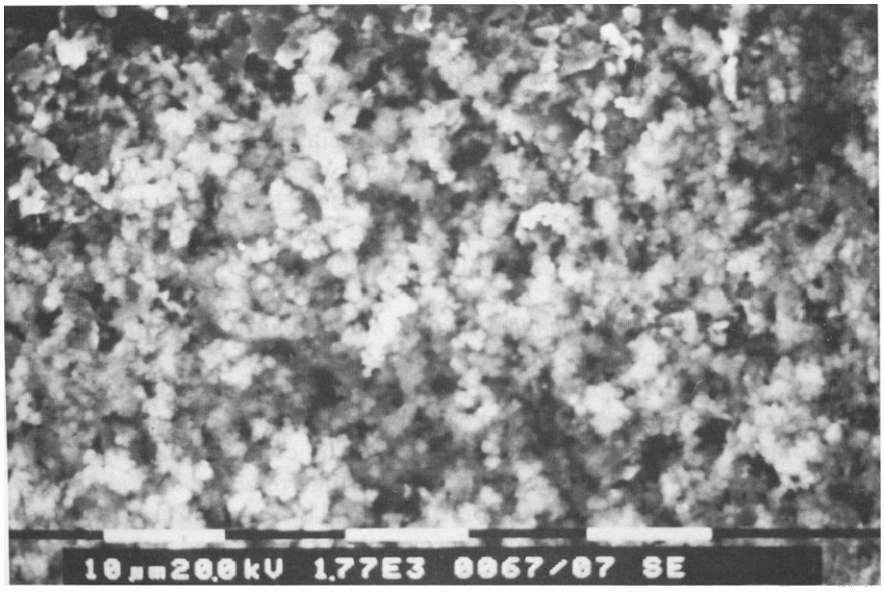

Fig. 3. Scanning electron micrograph of the continuous HIC polymer bed. The white bars represent a length of 10 μm. If the micrograph gives a true picture of the bed it is composed of "walls" of aggregated particles and "channels" between the aggregates in which buffer can flow. (Preparation and photo: Leif Ljung, Department of Anatomy, University of Uppsala, Uppsala, Sweden).

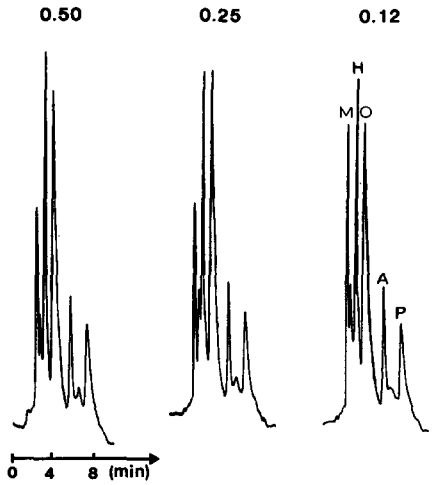

Fig. 4. Influence of flow-rate at constant gradient volume on the appearance of the chromatograms obtained by AEC on a continuous bed. The sample (40 μl) contained 20 μg each of the proteins myoglobin (M), haemoglobin (H), ovalbumin (O), albumin (A) and phycoerythrin (P). Bed dimensions, 38 × 6 mm I.D.; gradient, from 0 to 0.43 *M* sodium acetate in 0.01 *M* Tris–HCl (pH 8.5). A 5-ml gradient was used at the flow-rates 0.50, 0.25 and 0.12 ml/min. The figure shows that the resolution is independent of the flow-rate.

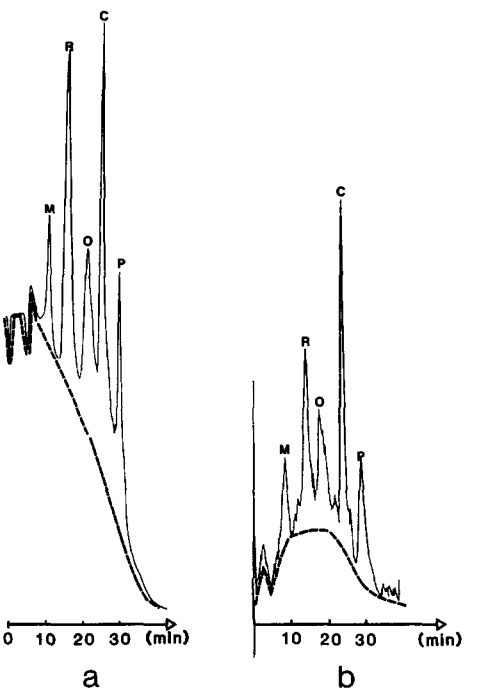

Fig. 5. Micro-column HIC with on-tube (a) and off-tube (b) detection. Bed dimensions, 70 × 0.3 mm I.D. The sample contained 0.4 μg each of the proteins myoglobin (M), ribonuclease (R), ovalbumin (O), α-chymotrypsinogen A (C) and phycoerythrin (P). Sample volume, 1 μl. A linear 35-μl gradient from 0.01 *M* sodium phosphate (pH 7.0), containing 2.25 *M* ammonium sulphate, to 0.01 *M* sodium phosphate (pH 7.0), containing 0.25 *M* ammonium sulphate, was used at a flow-rate of 1 μl/min. The principle of the on- and off-tube detection is given in Fig. 1. The syringe pump 10 (see Fig. 1) delivered 0.01 *M* sodium phosphate buffer (pH 7.0) at a flow-rate of 60 μl/min.

detector 9 consisted of a 30 × 0.15 mm I.D. fused-silica tubing 8 attached to the outlet of the micro-column 7 (see Figs. 1 and 5a). For off-tube monitoring, the flow-through cell (volume 8 μl; light path 10 mm) in the Bio-Rad Model 1306 HPLC UV detector (11 in Fig. 1) was used (Fig. 5b). The cross-flow solution, 0.01 *M* sodium phosphate (pH 7.0), was delivered from the syringe pump 10 at 0.06 ml/min.

RESULTS AND DISCUSSION

A comparison of Fig. 4 with Fig. 7a in ref. 11 indicates that the resolution of proteins on compressed continuous beds is about the same as that on compressed beds of the high-resolving non-porous agarose beads [2,6,7,11]. In view of this high resolution of the continuous beds, in combination with the very low cost and ease of preparation (which also makes them attractive for fractionation on a large scale), some readers may be interested in using such beds. Therefore, we have described in detail both the preparation and the handling of the columns. Studies in progress are aimed at further improvement of the chromatographic properties.

Fig. 3 indicates that the continuous beds are built up of "walls" of aggregated polymer particles (diameter *ca.* 0.5 μm) and "channels" (diameter 3–4 μm) between the aggregates. However, some caution is warranted in deducing the structure of the bed from the photo, since the preparation of the bed for microscopy may have changed its appearance. For instance, the observed "channel" diameter may be larger than the actual one [9,10].

Since the "walls" of the "channels" do not permit passage of proteins (Fig. 2) it is not surprising that the beds, after compression, resemble the compressed non-porous agarose beds [6–8] in the sense

that the resolution at constant gradient volume is independent of the flow-rate or even increase with an increase in flow-rate (see Fig. 4 in this paper and Fig. 1 in ref. 1). This characteristic and highly desirable feature will be discussed in a forthcoming paper.

The addition of ammonium sulphate to the monomer solution served to increase the hydrophobic interaction between the polymer chains formed and thus to create "channels" between them (the monomer should not be too hydrophilic). There are, however, other possible ways to design continuous beds, involving, for instance, the use of (mixtures of) organic solvents to fit the polarity (hydrophobicity) of the monomers and the polymers formed. By carefully tuning the experimental conditions it is undoubtedly possible to prepare more rigid and homogeneous continuous beds than those described herein and in ref. 1, thus increasing the efficiency of a column.

ACKNOWLEDGEMENTS

This study was financially supported by the Swedish Natural Science Research Council, and the Carl Trygger and the Knut and Alice Wallenberg Foundations.

REFERENCES

1 S. Hjertén, J.-L. Liao and R. Zhang, *J. Chromatogr.*, 473 (1989) 273.
2 S. Hjertén, in M. T. W. Hearn (Editor), *HPLC of Proteins, Peptides and Polynucleotides*, VCH Publishers, in press.
3 S. Hjertén, in H. Hirai (Editor), *Electrophoresis '83*, Walter de Gruyter, Berlin, 1984, pp. 71–79.
4 S. Hjertén and M.-D. Zhu, in B. Rånby (Editor), *Physical Chemistry of Colloids and Macromolecules, Proceedings of the Svedberg Symposium, Uppsala, August 22–24, 1984*, Blackwell, London 1987, pp. 133–136.
5 T. Takeuchi, T. Saito and D. Ishii, *J. Chromatogr.*, 351 (1986) 295.
6 S. Hjertén and J.-L. Liao, *J. Chromatogr.*, 457 (1988) 165.
7 J.-L. Liao and S. Hjertén, *J. Chromatogr.*, 457 (1988) 175.
8 S. Hjertén, K. Yao and J.-L. Liao, *Makromol. Chem., Macromol. Symp.*, 17 (1988) 349.
9 J. Gressel and A. W. Robards, *J. Chromatogr.*, 114 (1975) 455.
10 R. Rüchel and M. D. Brager, *Anal. Chem.*, 681 (1975) 415.
11 S. Hjertén, J. Mohammad, K.-O. Eriksson and J.-L. Liao, *Chromatographia*, 31 (1991) 85.