*Journal of Chromatography*, 639 (1993) 33–39
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 884

# Continuous buffer exchange of column chromatographic eluates using a hollow-fibre membrane module

Michael Kaufmann*, Thomas Schwarz and Peter Bartholmes

*Institute of Biochemistry, University of Witten/Herdecke, Stockumer Strasse 10, W-5810 Witten (Germany)*

ABSTRACT

A method was developed to remove continuously the concentration gradient of any low-molecular-mass substance in the eluate during adsorption liquid column chromatography. For this purpose, a hollow-fibre membrane module (HFMM) was directly connected to the outlet of the chromatographic column and on-line buffer exchange was achieved by cross-flow ultradialysis. It could be demonstrated that the HFMM when used during liquid chromatography has some additional advantages. It is an ideal tool to concentrate continuously column eluates by dialysis against polyethylene glycol solutions. Moreover, it is also possible to detect specifically enzyme activities on-line using an HFMM.

## INTRODUCTION

When liquid column chromatography is performed for biochemical purposes, it is often indispensable to exchange the buffer, reduce the volume or determine enzymatic activities of the eluate obtained. All these procedures are batch techniques which often cause many inconveniences or problems.

Interaction chromatography in principle requires alteration of the buffer composition during the elution phase in order to desorb bound macromolecules [1]. For that purpose, the concentration of low-molecular-mass substances (protons, salts or competitors) is altered in most of the applications such as affinity, ion-exchange or hydrophobic interaction chromatography [2]. The presence (or absence) of those desorbants in the eluate may cause the following disadvantages. Detection of separated macromolecules after or during chromatography by UV–Vis spectrophotometry can be impaired [3]. Distorted band patterns, such as pinching or flaring of lanes, can be caused by salts in the samples prior to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) [4]. Activity measurements are often disturbed or even rendered impossible when not performed at well defined salt concentrations [5]. Further, the stability of several macromolecules strictly depends on defined salt or buffer compositions [6]. In the literature there are several examples of the necessity for buffer exchange after chromatographic steps. Buffers were altered by dilution [7], tube dialysis [8] or molecular sieve chromatography using PD 10 columns (Pharmacia) [9]. As these methods are batch procedures, it may be necessary to exchange the buffer of every single fraction after column chromatography has been performed. Further, performing enzymatic assays with every fraction is very time consuming. In many instances, sample concentration is regarded as useful.

In order to overcome all these problems, we developed a method to exchange the buffer

* Corresponding author.

0021-9673/93/$06.00 

continuously, measure enzymatic activities or reduce the volume of the eluate during liquid column chromatography by cross-flow ultradialysis, using a hollow-fibre membrane module (HFMM) connected directly to the outlet of the column.

## EXPERIMENTAL

### *Chemicals*

Lactate dehydrogenase (LDH), bovine serum albumin (BSA), reduced nicotinamide adenine dinucleotide (NADH) and pyruvate were purchased from Boehringer (Mannheim, Germany) and polyethylene glycol 20 000 was obtained from Fluka (Buchs, Switzerland). All other chemicals used were of analytical-reagent grade appropriate for biochemical use. Water purified with a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout and all experiments were performed at room temperature.

### *Hollow-fibre membrane module*

All experiments were performed using an HFMM packed with Cuprophan C1 capillaries, which was a generous gift from Akzo (Obernburg, Germany) and is now commercially available from the authors. The specifications of the module are given in Table I.

TABLE I

SPECIFICATIONS OF THE HFMM

| Parameter | Value |
|---|---|
| Module length | 270 mm |
| Capillary length | 230 mm |
| Active capillary length | 215 mm |
| Case diameter | 10 mm |
| Packing diameter | 8 mm |
| Packing cross-section | 50 $mm^2$ |
| Module fill-grade | 29.7% |
| Capillary I.D. | 0.2 mm |
| Capillary O.D. | 0.222 mm |
| Number of capillaries | 50 |
| Molecular mass cut-off value | 5 kDa |
| Total intracapillary surface area | 68 $cm^2$ |
| Total extracapillary surface area | 75 $cm^2$ |
| Intracapillary (void) volume | 0.34 ml |

### *Conductivity measurements and volume determinations*

The efficiency of cross-flow ultradialysis in the HFMM was determined by measuring the decrease of intracapillary (IC) NaCl concentration using a flow-through conductivity electrode. For sample volume reduction experiments, 0.715 mg/ml BSA was used IC, 20% (w/w) polyethylene glycol 20 000 was used in the extracapillary (EC) space and the IC volume reduction was monitored by determining the increase in BSA concentration in the IC eluate according to Bradford [10].

### *Anion-exchange chromatography*

In order to measure the peak broadening caused by the void volume of the HFMM, anion-exchange chromatography was performed using Q-Sepharose fast flow (Pharmacia, Uppsala, Sweden) in an 8 × 1 cm I.D. column (Amicon, Witten, Germany). A 500-$\mu$g amount of LDH was applied in 1 ml of 50 m*M* triethanolamine (TEA) (pH 7.8) and 30 ml of a linear NaCl gradient (0–1 *M*) were run at 1 ml/min in order to desorb bound protein.

### *Covalent chromatography*

To give an example of the extreme detection problems caused by low-molecular-mass light-absorbing molecules, we performed covalent chromatography using thiol-Sepharose 4B (Pharmacia). A column (8 × 1 cm I.D.) (Amicon) was equilibrated with 50 m*M* TEA (pH 7.8) and loaded with 400 $\mu$l of a crude cell extract (5 mg/ml) prepared from *Euglena gracilis* as described previously [11]. Covalently bound sample (200 $\mu$g) was removed from the column using 30 ml of a linear gradient (0–50 m*M*, 0.2 ml/min) of $\beta$-mercaptoethanol in the same buffer.

### *On-line detection of enzymatic activity*

In order to show the possibility of using the HFMM as a sensor for detecting enzymatic activity in a continuous buffer flow, we selected the reduction of pyruvate to lactate, as catalysed by LDH [12]. Potassium phosphate (50 m*M*, pH 7.5) containing 3.1 m*M* pyruvate plus 1.5 m*M* NADH was circularly pumped EC in a total volume of 250 ml at 1 ml/min. A sample of 5 $\mu$g

of LDH in 100 μl was injected during a continuous IC flow of 0.8 ml/min of 50 m*M* potassium phosphate (pH 7.5). LDH activity was detected spectrophotometrically by measuring the decrease in NADH at 340 nm in a 1-mm flow-through quartz cuvette.

## RESULTS

### *On-line desalting and buffer exchange*

As can be seen in Fig. 1, the efficiency of buffer exchange depends on both the EC and IC flow-rates. Using an IC buffer flow-rate of 0.5–1.0 ml/min, a flow-rate usually applied for fast protein liquid chromatographic (FPLC) experiments and an EC buffer flow of 30 ml/min, the efficiency of desalting a 1 *M* NaCl solution is better than 95%.

### *Peak broadening*

The effect of peak broadening caused by passing the IC void volume of the HFMM is demonstrated in Fig. 2. A sample of LDH was separated using a column packed with Q-Sepharose fast flow either with or without the HFMM. Peaks were delayed by *ca.* 1 ml in the case of desalting the eluate with the HFMM but peak broadening was below 20% (half-width of the main LDH peak). The partial disappearance of the very first small peak could be due to low-molecular-mass compounds being eliminated by the HFMM.

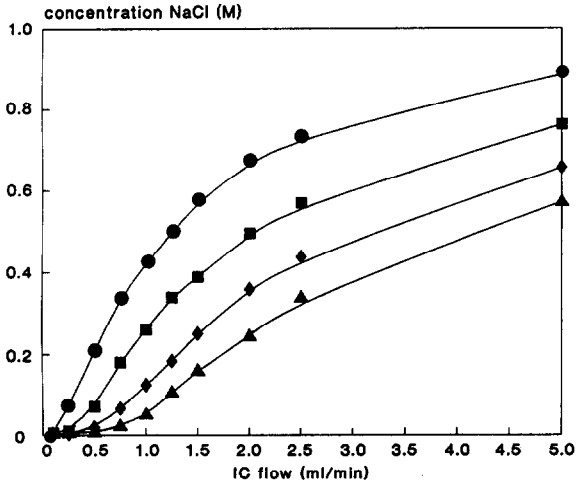


Fig. 1. Efficiency of desalting 1 *M* NaCl at EC flow-rates of (●) 1, (■) 5, (◆) 15 and (▲) 30 ml/min.

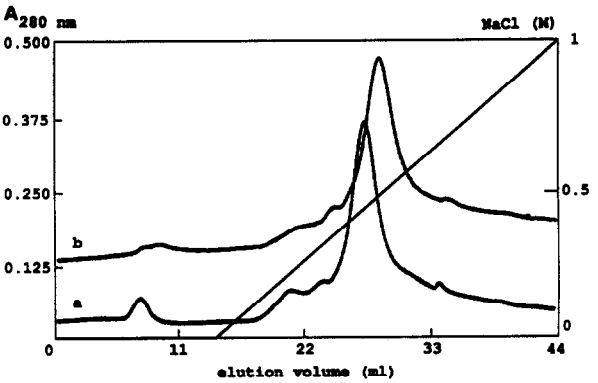


Fig. 2. Anion-exchange chromatography of LDH using a Q-Sepharose fast flow column either (a) without or (b) with desalting of the eluate at a flow-rate of 5 ml/min (EC).

### *Improvement of detection*

In many applications of liquid column chromatography, continuous detection of macromolecules in the eluate by measuring the absorbance is interfered with or is even impossible because of the presence of light-absorbing low-molecular-mass substances. In order to demonstrate the ability to overcome such detection problems, covalent chromatography using thiol-Sepharose [13] was performed with a crude cell extract prepared from *Euglena gracilis*. As shown in Fig. 3a, detection of proteins at 280 nm during chromatography is impossible owing to 2-thiopyridyl groups replaced by both protein binding to the column and thiol-reducing agents during elution. In addition, a baseline shift due to the absorption of β-mercaptoethanol at 280 nm occurs. By using the HFMM during chromatography (Fig. 3b) it was possible to detect two small protein peaks without any baseline shift. The absence of those peaks in the control experiment without injecting a sample indicates that the 2-thiopyridyl groups indeed were completely removed by cross-flow ultradialysis under the described experimental conditions. The first peak, eluting during sample injection, should contain both non-bound proteins and 2-thiopyridyl groups replaced by bound protein. Therefore, protein determination by peak inte-

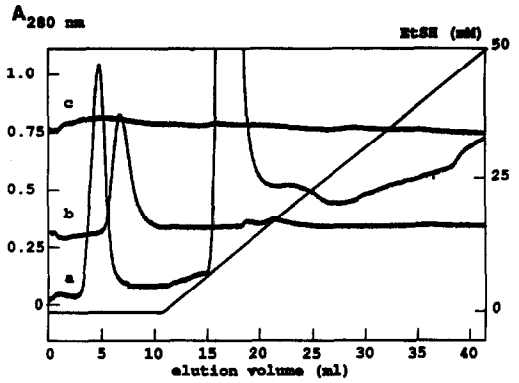


Fig. 3. Covalent chromatography of a crude cell extract derived from *Euglena gracilis* using a thiol-Sepharose column. Sample was eluted with 30 ml of a linear gradient of β-mercaptoethanol (0–50 m*M*). (a) Conventional chromatography; (b) chromatography using the HFMM with an EC flow-rate of 30 ml/min; (c) control, performed as in (b) but without sample.

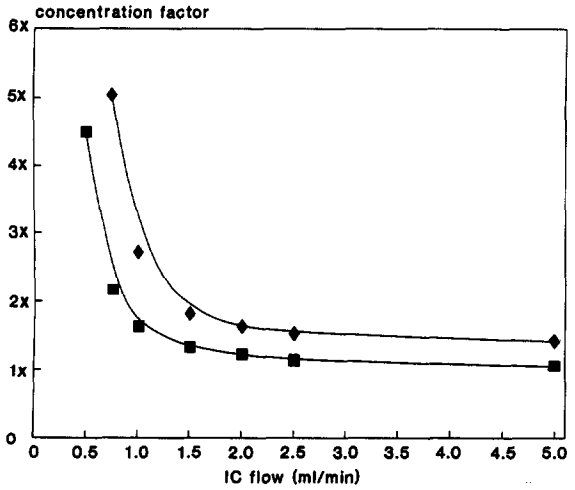


Fig. 4. Concentration of 0.715 mg/ml BSA IC using 20% polyethylene glycol 20 000 at EC flow-rates of (■) 1 and (◆) 5 ml/min.

gration is not possible. The total area under this peak decreases when the HFMM is used, indicating that 2-thiopyridyl groups are now removed, and consequently the amount of protein can be determined.

*On-line concentration*

Installing the HFMM between the outlet of the column and the detection unit not only solves desalting problems. Using hygroscopic solutions EC, it should be possible to create osmotic pressure gradients useful for concentrating solutions of macromolecules by IC volume reduction. In Fig. 4 the capability of such an arrangement is demonstrated with a BSA solution IC and 20% (w/w) polyethylene glycol 20 000 EC. As can be seen, we were able to concentrate up to fivefold, but the efficiency to concentrate proteins is not limited in principle, *e.g.*, by the choice of flow-rates, but rather by IC protein precipitation, membrane adsorption or inaccuracy of pump velocity, responsible for exact and constant IC flow-rates.

*On-line detection of enzymatic activity*

The HFMM also appeared to be suitable for detecting enzymatic activities within a column eluate. Consequently, a low-molecular-mass substrate was pumped continuously EC and transferred into the IC and conversion was detected by measuring the reaction product spectrophotometrically. When an NADH–pyruvate-con-

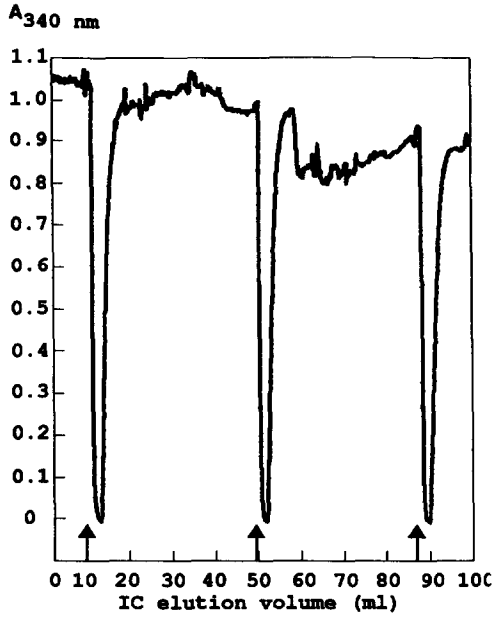


Fig. 5. On-line detection of LDH activity: 50 m*M* potassium phosphate (pH 7.5) containing 3.1 m*M* pyruvate and 1.5 m*M* NADH was pumped circularly EC. The NADH decrease was detected spectrophotometrically at 340 nm directly at the outlet of the HFMM. Arrows indicate injection of 5 μg LDH per 100 μl into a continuous IC flow-rate of 0.8 ml/min.

taining buffer was used EC and small amounts of LDH were injected into a continuous IC flow, clearly negative peaks indicating the corresponding NADH turnover were detectable (Fig. 5). No significant positive protein peaks were found at 280 nm with the same sensitivity (data not shown). The continuous, slow decrease of the baseline is caused by the loss of NADH, as the EC flow was pumped circularly in order to recycle and thus minimize the NADH–pyruvate consumption.

## DISCUSSION

Neither the principle of constructing HFMMs nor their application in order to perform cross-flow ultradialysis is new. However, pumping a continuous sample flow, which contains sequentially appearing different macromolecules through a small IC space and obtaining the same pattern within the flow-through after passing an HFMM, has not previously been reported. Moreover, combining this newly discovered principle of application with liquid chromatographic procedures, by connecting HFMMs to the outlet of chromatographic columns, appeared to provide an advance for liquid chromatography in general: the method allows desalting, concentration and enzymatic activity detection of column eluates to be performed on-line without significantly altering the separation profiles.

Hydrophobic interaction, ion exchange, hydroxyapatite and some applications of affinity chromatography essentially are accompanied by the formation of salt gradients in the eluate. There are several circumstances that make desalting of such a column eluate indispensable. It is well known that SDS-PAGE requires samples that contain low concentrations of salt. On-line desalting therefore makes it possible to avoid separately desalting every single fraction collected after liquid column chromatography, when subsequent SDS-PAGE analysis is planned. During purification procedures, in many instances desalting steps are necessary to achieve protein binding to matrices of further chromatographic columns. On-line desalting is therefore suitable for saving as much time as possible, which is often a crucial condition when sensitive proteins are to be purified. In addition, detection problems that often occur when continuous-flow absorbance spectrometry is performed, *e.g.*, with light-absorbing salts or low-molecular-mass compounds present in a column eluate, can be obviated by on-line desalting. Free-flow electrophoresis (FFE), a method which requires very low ionic strengths, is capable of continuously separating biomolecules during purification procedures [14]. Thus on-line desalting over an HFMM is a helpful tool prior to FFE. Not only is desalting of the column eluate a useful application, but also the transfer of salts into the eluate in order to increase the ionic strength. It is known that the proteins derived from halophilic bacteria require high concentrations of salt, which makes the application of salt-dependent purification steps such as ion-exchange chromatography impossible [5]. Lanyi [15] reported that the deactivation of such proteins by exposure to low salt concentrations is a time-dependent process. Two HFMMs, the first connected to the inlet of the column in order to desalt the sample prior to ion-exchange chromatography and the second connected to the outlet to increase again the ionic strength should minimize the exposure time at low ionic strengths and therefore may solve some purification problems.

The use of on-line HFMM applications is not limited to buffer exchange designs. Another time-consuming step during purification procedures is the reduction of buffer volume. Therefore, mostly batch procedures made use of ammonium sulphate precipitation [16], ultrafiltration [17,18] or lyophilization [19]. As demonstrated above, an HFMM pumped with polyethylene glycol EC is sufficient to concentrate macromolecules on-line without simultaneously concentrating low-molecular-mass compounds, as occurs during lyophilization. We used polyethylene glycol 20 000 in a 20% (w/w) solution (non-saturating conditions) without solubility or viscosity problems. This method allows the fractionation of column eluates after concentration and further applies simultaneously to the chromatographic procedure without consuming additional time. The resolving power of gel filtration depends strictly on the sample volume and

consequently on-line concentration should in general be useful prior to molecular sieve chromatography. It is known that the flatter the salt gradient selected in absorption chromatography, the more the separation of peaks is improved but the concentration of macromolecules in the eluate is decreased. Therefore, one would expect better overall separations by running flat salt gradients and compensating for the indispensable eluate dilution by applying an HFMM to reconcentrate continuously the eluate obtained.

In order to purify enzymes, it is necessary to determine the enzymatic activity after each chromatographic step. In most instances many fractions have to be assayed. There are approaches in the literature for mechanizing this detection procedure by flow-injection analysis [20]. Nevertheless, 2 min are required for the determination of each data point in the case of quasi-on-line detection of LDH. In contrast, the properties of an HFMM used on-line as mentioned above give the opportunity to detect enzymatic activity and record a continuous enzyme elution profile. In combination with a flow-through detection of protein concentration at 280 nm and a suitable computer program, it should be possible to record the specific activity during chromatography and thereby to optimize any automatic collection of fractions.

It should be noted that the three principles of using HFMMs in the mode described here (buffer exchange, concentration and enzyme detection) can also be combined. For example, a column eluate containing a highly diluted enzyme can be concentrated on-line by a first HFMM with the activity being detected continuously by a second HFMM. Another example of combining more HFMMs could be the preparation of pyridoxal 5′-phosphate-dependent apoenzymes, *e.g.*, tryptophan synthase [21]. The first HFMM directly mounted on the last chromatographic column of a purification procedure could be used for detecting enzymatic activity, the second to transfer hydroxylammonium chloride into the IC in order to obtain apoenzymes and a third to remove the resulting oxime of the reaction.

The principle of cross-flow ultradialysis depends on pore size, molecular mass of sample compounds, temperature, total membrane surface and EC and IC flow rates. When HFMMs are used in the manner described here, they must obey a new, crucial principle of construction: in comparison with conventional HFMMs, an on-line desalting HFMM should have an increased ratio of capillary length to total number of capillaries. This principle of construction is necessary because the production process of HFMMs causes significant deviations of the fibre inner diameters directly at the inlet and outlet of the module, implying different flow-rates in the capillaries and consequently peak broadening. Further, the longer the capillaries are, the more the concentration gradient is extended and therefore the more the efficiency of dialysis is increased. It is also important to minimize the void volume, because of sample dilution and consequent peak broadening. On the other hand, minimizing the void volume implies a reduction in the total exchange surface and therefore a decrease in efficiency. This disadvantage can be compensated for by warming the EC buffer, as the time of sample exposure in the IC normally is very short.

In this study, an HFMM suitable for FPLC as well as HPLC applications and standard chromatography using columns with matrix volumes not more than 10 ml was investigated. The only minor restrictions of using HFMMs during column chromatography are peak broadening caused by the void volume of the IC space and velocity limitations in sample flow. As we could demonstrate, the HFMM works with sufficient efficiency at the flow-rates usually used for FPLC or standard chromatography with columns of the described sizes. The peak broadening was below 20% and therefore readily tolerable. Consequently, the use of HFMMs should become an advance in column chromatography in general and HFMMs of different sizes can be used for any scale of chromatographic columns.

ACKNOWLEDGEMENT

We thank Dr. G. Eberhard (Akzo) for providing the HFMMs used in this study.

REFERENCES


1 R. Scopes, in C.R. Cantor (Editor), *Protein Purification*, Springer, New York, 1982, Ch. 4, p. 85.
2 S. Roe, in E.L.V. Harris and S. Angal (Editors), *Protein Purification Methods*, IRL Press, Oxford, 1989, Ch. 4, p. 193.
3 D. Freifelder, *Physical Biochemistry*, Freeman, New York, 1982, Ch. 5, p. 500.
4 D.E. Garfin, *Methods Enzymol.*, 182 (1990) 425–441.
5 W. Leicht and S. Pundak, *Anal. Biochem.*, 114 (1981) 186–192.
6 M. Mevarech and E. Neumann, *Biochemistry*, 16 (1977) 3786–3792.
7 S.S. Patel, A.H. Rosenberg, F.W. Studier and K.A. Johnson, *J. Biol. Chem.*, 267 (1992) 15 013–15 021.
8 R.M. Luche, W.C. Smart and T.G. Cooper, *Proc. Natl. Acad. Sci. U.S.A.*, 89 (1992) 7412–7416.
9 M. Kaufmann, T. Schwarz, R. Jaenicke, K.D. Schnackerz, H.E. Meyer and P. Bartholmes, *Biochemistry*, 30 (1991) 4173–4179.
10 M.M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
11 J. Li and J.A. Schiff, *Biochem. J.*, 274 (1991) 355–360.
12 A. Denicola-Seoane and B.M. Anderson, *J. Biol. Chem.*, 265 (1990) 3691–3696.
13 A.C. de Graaf-Hess and A. de Kok, *FEBS Lett.*, 143 (1982) 261–264.
14 S. Nath, H. Schutte, G. Weber, H. Hustedt and W.D. Deckwer, *Electrophoresis*, 11 (1990) 937–941.
15 J.K. Lanyi, *Bacteriol. Rev.*, 38 (1974) 272–290.
16 D. Schopohl, A. Müller-Taubenberger, B. Orthen, H. Hess and W. Reutter, *J. Biol. Chem.*, 167 (1992) 2400–2405.
17 T. Vernet, H.E. Khouri, P. Laflamme, D.C. Tessier, R. Musil, B.J. Gour-Salin, A.C. Storer and D.Y. Thomas, *J. Biol. Chem.*, 266 (1991) 21 451–21 457.
18 M. Burgos-Trinidad, R. Ismail, R.A. Ettinger, J.M. Prahl and H.F. DeLuca, *J. Biol. Chem.*, 267 (1992) 3498–3505.
19 A. Sarnesto, T. Köhlin, O. Hindsgaul, K. Vogele, M. Blaszczyk-Thurin and J. Thurin, *J. Biol. Chem.*, 267 (1992) 2745–2752.
20 M. Tabata, M. Totani and T. Murachi, *Anal. Biochem.*, 193 (1991) 112–117.
21 P. Bartholmes, H. Balk and K. Kirschner, *Biochemistry*, 19 (1980) 4527–4533.