ELSEVIER

Journal of Chromatography B, 763 (2001) 107–120

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Chromatographic performance of a thin microporous bed of nitrocellulose

Maria Lönnberg[a,b,*], Jan Carlsson[a,b]

[a]*Center for Surface Biotechnology, Box 577, Uppsala University, S-751 23 Uppsala, Sweden*
[b]*Pharmacia Diagnostics AB, S-751 82 Uppsala, Sweden*

Received 5 June 2001; received in revised form 8 August 2001; accepted 8 August 2001

Abstract

Chromatography along thin (125 μm) porous beds of nitrocellulose, layered on top of an polyester backing, shows good separation efficiency with plate heights of 10–20 μm. Flow is controlled by capillary forces and shows low rate variations between the individual disposable devices. Positively charged groups were introduced into the nitrocellulose and efficient separation of transferrin isoforms, differing by only 0.1 p*I* units, was found after a short migration distance (1 cm). The upper surface is not covered, which allows sample and reagents to be added, and the clear backing permits detection. The chromatography can easily be combined on-line with sensitive immunoassay detection down to the p*M* (10^{-12} *M*) range. This microscaled combination device should have a wide range of applications in analytical biochemistry.

Keywords: Thin microporous beds; Lateral flow; Nitrocellulose; Polyethylenimine

1. Introduction

Microanalytical liquid chromatography has been developed during the last few years for use in combinatorial chemistry, proteomics and clinical chemistry. So far the miniaturisation has focused mainly on the size of the columns, while maintaining ancillary equipment, such as pumps and detectors, in traditional sizes. However, demands for an increasing number of tests create a need for rapid parallel processing and also a need to use small sample volumes of the valuable biological materials. The ideal system should preferably perform high-resolution separation in combination with sensitive detection in a self-contained disposable unit without any need for external regulation. Such labs-on-a-chip could then be used for automatic high-throughput sample analysis.

Membranes with a narrow distribution of pore size seem to offer the required properties of a suitable chromatographic material. In fact, ion-exchange and affinity chromatography in membranes is claimed to be more efficient and rapid than the corresponding separation methods based on porous particles. The mass transport is enhanced by the convective flow and mass transfer, due to pore and film diffusion, is minimised. [1–3]. Thus stacks of thin nylon mem-

*Corresponding author. Pharmacia Diagnostics AB, S-751 82 Uppsala, Sweden. Tel.: +46-18-166-089; fax: +46-18-166-465.
E-mail address: maria.lonnberg@pharmacia.com (M. Lönnberg).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00376-0

branes with small pore sizes (1–3 μm in diameter) have been shown to function well as ion-exchange chromatographic supports for fast and efficient analytical and preparative separation of proteins [4]. Ion-exchange separation of small molecules, such as oligonucleotides with different chain lengths, has been performed in some minutes with good separation efficiency in a 3 mm monolithic disc [5]. Rapid ion-exchange separations of peptides and the reversed-phase separation of a steroid mixture have also been achieved in a thin disc [6]. The equipment used for these separations in membranes has, however, been the same as for traditional particle-based techniques.

In the 1970s microchromatography *along* a membrane (rather than across the membrane) was introduced in order to miniaturise, simplify and speed-up analytical chromatographic techniques suitable for work with proteins, nucleic acids and other biological substances [7]. This interesting concept seems not to have been further developed, but in the 1980s the use of a rapid immunoassay technique based on flow along thin membranes was initiated. This technique came to be known as lateral chromatography or immunochromatography [8]. It is a rapid, easy-to-use test e.g for clinically interesting analytes in complex media such as plasma. The porous membrane material is mostly layered on top of a non-porous transparent backing to simplify the lateral liquid flow driven by capillary forces. The upper surface can be partially open to allow sample and reagents to be added. The test sample migrates through an affinity zone where immobilised antibodies capture the analyte. After flowing labelled antibodies through the zone, a sandwich complex is formed which can be detected by eye or with suitable detection equipment. This approach gives tests with high specificity and sensitivity. For instance, a rapid quantitative immunochromatographic test for IgE has been described with a detection limit of 0.13 p*M* IgE when using carbon black as label and a flat-bed scanner as detection equipment [9].

However, almost every protein shows some heterogeneity and it seems to be of genuine clinical significance to measure the different isoforms [10–12] that mostly can not be distinguish solely by immunoassays. Recently, we found that isoforms of transferrin could be rapidly analysed [13] by the combination of isocratic ion-exchange chromatography on-line with immunochromatographic detection in a nitrocellulose membrane device. The obtained results, with high resolution between the different transferrin isoforms, in combination with the above-mentioned possibilities for on-line detection, encouraged us to further examine the chromatographic performance of the used nitro–cellulose membrane. Membranes for ion-exchange or affinity chromatography should fulfil some important criteria such as having uniform structure, narrow pore size distribution, high hydrophilicity and low nonspecific protein adsorption [4]. In addition, good chemical and mechanical resistance is needed, and it should also be possible to provide the membranes with suitable ligands for the planned type of chromatography, without changing the above properties. In most of these respects nitrocellulose offers the required properties but the protein adsorption needs to be reduced when used for separation of substances at low concentration.

The present study is evaluating a thin-bed chromatographic separation technique performed in disposable nitrocellulose membranes. The principle for this type of chromatography is illustrated in Fig. 1.

In conventional chromatographic separation the mobile phase flow is normally maintained and regulated by pumps. However, when working with microscaled chromatography in open systems other flow regulating principles are needed. Here, the

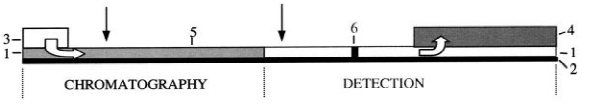

Fig. 1. Device for thin-bed chromatography. The device is composed of a membrane with separation, transport and detection zones (1). The membrane has an non-porous backing (2) on the bottom side but its upper side is open for application of reagents. The driving force for the migration of liquid is capillarity, and the flow is kept up by a buffer-delivering pad (3) and an absorbent sink (4). The separation zone (5) can be equipped with different functionalities for the desired type of chromatography e.g. ion-exchange, affinity or hydrophobic chromatography. The detection of the separated molecules can be performed by immunoassay, using an immunoaffinity zone (6), which together with a labelled reactant gives a sensitive quantitative determination of the target molecules. Other commonly utilised detection methods, such as staining of the separated molecules, can also be used.

capillarity driven flow behaviour and the potential for flow-rate regulation are being examined. The thin and flat membrane, with its transparent backing, makes it possible to monitor the zone broadening process by means of a flat-bed scanner, as the separation progress along the membrane. Positively charged groups have been introduced to the microporous structure to yield a hybrid matrix with unique properties, ideal for chromatography. The resolution obtained in the anion-exchange membrane is tested by the separation of nanogram quantities of ^{125}I-labelled proteins with varying p*I*.

2. Materials and methods

The water used in all experiments was purified using a Milli-Q Academic (Millipore, MA, USA) equipment.

2.1. Nitrocellulose membrane

The tested membrane was a 125 μm (110–140 μm) thick microporous nitrocellulose membrane with 3, 5 or 8 μm nominal pore size diameter, backed with a 100 μm thick optically clear (for visible light) polyester film, impervious to water (Whatman International Ltd, Maidstone, UK, www.whatman.com).

2.2. Absorbent sinks

A blotting cellulose paper GB 004, (Schleicher & Schuell GmbH, Dassel, Germany, www.s-und-s.de) was used as an absorbent sink in most of the experiments.

Glass cellulose mixture paper WF 1.5, cellulose blotting and chromatography paper grade 17 Chr (Whatman International Ltd, Maidstone, UK), cellulose absorbent paper Gelman 165 and 197 (Pall Corporation, www.pall.com) were also tested.

The sinks were mounted on the separation or detection membrane (overlapping 0.5 cm) and attached to the backing and to the membrane by an adhesive tape.

2.3. Buffer reservoir pad

A buffer reservoir pad in the form of a polyvinyl alcohol sponge with 60 μm pore size (Kanebo Ltd, Tokyo, Japan) was used throughout. This material was washed and dried before use and its liquid holding capacity was estimated to 0.69 ml/cm^2 and the pad area was chosen to significantly exceed the volume of mobile phase needed during the separation.

2.4. Purified proteins and ^{125}I-labelling of proteins

The isolation and pI estimation of tetrasialotransferrin (p*I* 5.32), trisialotransferrin (p*I* 5.46), disialotransferrin (p*I* 5.56) and bovine serum albumin (BSA, p*I* 4.8) has been described elsewhere [13] together with the labelling procedure.

2.5. Detection

An Arcus II scanner (Agfa) with a true optical resolution of 600×1200 pixels per inch (ppi) and a 12 bit sample depth for greyscale (4096 levels) was used in the reflection mode to determine the location and colour distribution for zones to be detected.

2.6. Measurement of the inner volume in the membrane

By applying 5 μl of a 1 mg/ml carbon black suspension in 5 m*M* borate buffer with 1% Tween 20 it was possible to easily measure the spreading area in the membrane.

The porosity was estimated by comparing the dry and the wetted weight after immersion in a buffer.

2.7. Measurement of the flow

The flow-rate was typically measured in a buffer containing 20 m*M* bis-tris, pH 6.3 and 0.1% Tween 20. Strips of nitrocellulose membranes, 1 cm wide, were mounted with a 1×3 cm absorbent sink and a 1×1 cm of buffer pad. The time measurement was started when the liquid front or a bromophenol blue

(BFB) marker zone had migrated 1 cm and was read every 1 cm up to 6 cm.

2.8. Estimation of the plate height for nitrocellulose membrane

The non-substituted nitrocellulose membrane strips (3 and 8 μm nominal pore size, 7×2 cm) were mounted with an absorbent sink (GB004, 3×2 cm) and wetted with an elution buffer containing 20 m*M* bis-tris pH 6.3 and 0.1% Tween 20 by applying a buffer pad on the free end. A thin line of BFB was placed on the membrane by softly dipping the free short end of the membrane, first on a pad with BFB (2 mg/ml with 0.1% Tween 20) and then on a pad with elution buffer. The membrane was then immediately placed in the scanner with the backing towards the glass, and the refilled buffer pad was placed on the free end of the membrane (up to 0.5 cm) some mm upstream of the BFB band. The time measurement and the scanner were started when BFB had migrated 1 cm on the membrane and new images were taken as the zone was passing the 2, 3, 4 and 5 cm marks. The images were converted to digital values and the intensity of the resulting peak from the BFB distribution was plotted against the migration distance. The peak width at 0.607 of the peak height were measured. This corresponds to $2\times\sigma$ (σ=1 SD) of a Gaussian distribution curve [14]. The plate height was then calculated as $H = \sigma^2/X$, where X is the migration distance.

2.9. Preparation of the anion-exchange membrane by adsorbing polyethylenimine (PEI)

The PEI solution (Sigma, MO, USA) was weighed, added to Milli-Q water and stirred for more than 1 h. The pH was measured and was typically 10.3 for 0.03% and 11.1 for 2.7% PEI. The nitrocellulose membrane, cut into 4×21 cm, was carefully lowered into the PEI solution (typically 400 cm^2 of membrane/100 ml liquid) and agitated for 3 h. The membrane was then wiped and shaken for another 0.5 h in 0.1% Tween 20 (Sigma, MO, USA). The membranes were finally dried at room temperature for some hours and stored at +4°C in a plastic bag.

2.10. Determination of introduced primary and secondary amino groups in the nitrocellulose membrane

The nitrocellulose membranes with adsorbed PEI were cut into 25 mm^2 pieces and each was placed in a test tube (10 replicates). An aliquot of 0.3 ml of freshly prepared 4 m*M* sulfo-succinimidyl-3-(4-hydroxyphenyl) propionate (sulfo-SHPP) (Pierce, Rockford, IL, USA) dissolved in 0.1 *M* $NaHCO_3$ pH 8.3, 3% Triton X-100 (Sigma) was added to the tubes. The tubes were gently shaken for 0.5 h at room temperature before washing the pieces repeatedly with water and 0.25 *M* $Na_2CO_3/NaHCO_3$ pH 11.5. An aliquot of 0.3 ml of the BCA working reagent (BCA, Protein assay reagent, Pierce) was added to the membranes. As a reference, 10 μl standard solution of Tyrosine–HCl (Sigma) (0.078–10 m*M*) was used. The tubes were incubated at +37°C for 2 h and finally cooled down to +4°C (0.5 h), 0.2 ml sample were taken from each tube, transferred to microplate wells, and measured at 540 nm in a spectrophotometric microplate reader (Multiscan, Labsystem Oy, Helsinki, Finland). The amount of reacted sulfo-SHPP in the membrane was calculated by comparing the obtained intensity with the intensity of the tyrosine standard by using a spline curve fit program (GraphPad Prism®, GraphPad Software, Inc, San Diego, CA, USA). The result was finally corrected for the low figures obtained when incubating the reagent with a non-substituted membrane.

2.11. Determination of introduced primary amino groups in the nitrocellulose membrane

2×50 mm^2 of the PEI membranes were shaken in 0.5 ml of 2 m*M* freshly prepared *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP, Pierce) in 0.1 *M* phosphate buffer pH 7.5 containing 1% Tween 20 during 0.5 h at room temperature [15]. The membranes were washed twice in 2 ml of 0.1 *M* phosphate buffer pH 7.5 containing 1% Tween 20. Pyridine-2-thione was released by shaking the membranes with 1.2 ml of 0.02 *M* dithiothreitol (Sigma) in 0.17 *M* phosphate buffer pH 8.5 during 1 h at room temperature. The absorbance at 343 nm was measured and the amount of released pyridine-2-

thione was calculated by using the molar extinction coefficient of 8.08×10^{3} M^{-1} cm^{-1}. The amount of primary amines was calculated after correcting for the low nonspecific binding to the non-substituted membrane.

2.12. Determination of albumin binding capacity for the anion-exchange membrane

The anion-exchange nitrocellulose membranes were cut into pieces (0.5×4 cm). One end was formed as a tip. The membranes were mounted with an absorbent sink (GB004) on the other end. The tip of the membrane was lowered into 20 μl of a buffer containing 10 m*M* tris pH 8.0 and 0.1% Tween 20 placed in microplate wells. After absorption, the membrane was moved to a well containing 20 μl of 10 mg/ml human albumin (Sigma) in 10 m*M* tris pH 8.0 and 0.1% Tween 20. Finally it was moved to a well containing 10 m*M* tris pH 8.0 and 0.1% Tween 20. To control that the albumin also could be released from the charged membrane some of the membranes were instead moved to wells containing 2 *M* NaCl, 10 m*M* tris pH 8.0 and 0.1% Tween 20 for the final step. The membranes were cut into pieces (25 mm^2) and the amount of bound albumin was tested with the BCA assay in accordance with 2.10 above, using 10 μl of 0.064–4 mg/ml human albumin as a reference.

2.13. Ion-exchange separation of ^{125}I-labelled proteins

The positively charged nitrocellulose membranes (5 μm) were cut into 0.5×4 cm pieces and mounted by an adhesive tape with 0.5×2 cm pieces of absorbent sink (GB004). One μl of the ^{125}I-labelled protein (0.5–3 μg/ml, diluted in a buffer containing 20 m*M* bis-tris pH 6.3–6.4, 0.1% Tween 20, 0.1 m*M* Fe(III) citrate, 1 m*M* $NaHCO_3$ and 0.05% NaN_3) was applied 1 cm from the free end of the membranes. The flow of elution buffer was then started by applying a buffer reservoir pad (0.5×1 cm) on the free end of the membranes. After the appropriate elution time the buffer pad was removed and the flow stopped. The membranes were then immediately cut into pieces (a) 0–2 cm, (b) 2–3 cm and (c) 3–4 cm (including the absorbent sink). Each piece was analysed in a gamma counter. Individual membrane strips were used for each protein and for every change in assay conditions.

The effect of variations in migration time is shown in Fig. 7. In this example a membrane incubated with 0.5% PEI (about 19 μmol PEI-N/cm^3obtained) was used. The elution buffer contained 20 m*M* bis-tris pH 6.30, 20 m*M* NaCl and 0.1% Tween 20. The proteins were ^{125}I-asialo transferrin, ^{125}I-tetrasialo transferrin and ^{125}I-BSA all with 1 mg/ml BSA added.

The effect of variations in ionic strength of the elution buffer is shown in Fig. 8. In this example a membrane incubated with 0.05, 0.1, 0.3 or 0.6% PEI (about 11, 13, 17, 20 μmol PEI-N/cm^3 obtained respectively) was used. The elution buffer contained 20 m*M* bis-tris pH 6.33–6.38, 0–40 m*M* NaCl and 0.1% Tween 20. The proteins used were ^{125}I-asialo transferrin, ^{125}I-disialo transferrin, ^{125}I-tetrasialo transferrin and ^{125}I-BSA all with 4 mg/ml BSA added.

3. Results and discussion

3.1. The nitrocellulose membrane

The nitrocellulose membrane used in the present work is manufactured as a cast on a thin polyester backing and is therefore easy to handle. Its network structure is shown in Fig. 2. It has a total volume of 12.5 μl/cm^2 with a measured porosity of 80–90%. A volume of 10–11 μl/cm^2 is thus available for liquid permeation. The dry membrane absorbs aqueous solutions (buffers) easily and quickly becomes homogeneously wetted (visually judged).

The liquid spreading on the nitrocellulose membranes was examined by applying small aliquots of a carbon black suspension on pieces of membranes. Under the mobile phase conditions used there was no visible adsorption of the chosen grade of carbon black used. The spreading pattern reflects the thickness and the porosity of the membrane and probably also the surface properties and can, thus, be used to measure the homogeneity of the membranes. The measured spreading area for 5 μl of an aqueous carbon black suspension on membranes produced

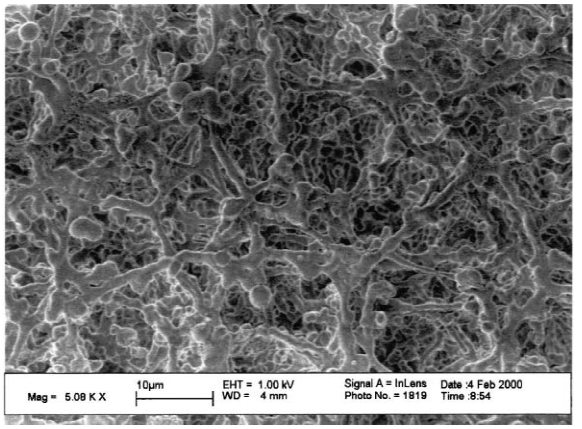

Fig. 2. The network structure of nitrocellulose membrane. The general appearance of the network indicates that liquid flow through the membrane should be of convective type and that mass transport limitations probably are due to film diffusion only. The digital image of the 3 μm (nominal pore size) membrane was taken in a LEO low voltage scanning electron microscope in a magnification of 5080× (Lahja Sevéus, Uppsala, Sweden).

during the period 1995–2000 was 58.5, 64.1 and 79 mm^2 respectively for the tested 3, 5 and 8 μm membrane and the variation between batches was about 11–15% expressed as coefficients of variation. The variation within one batch was about 3% for the membranes delivered during 1999. These results indicate that the homogeneity of commercially obtainable nitrocellulose membrane is acceptable for use in analytical thin-bed chromatography, where each separation is performed in an individual membrane device.

The described membrane analytical process, i.e. the combination with a sensitive immunoassay detection, makes possible specific determinations of very low concentrations (pM–nM) of substances, provided that the separation system can be optimised to avoid interference such as non-specific binding of the separating substances (proteins) on the nitrocellulose. Non-specific binding can be reduced by work in the presence of detergents and/or protecting proteins such as albumin. In the separation presented in this work Tween 20 was used for pretreating of the PEI membrane, as well as in the sample-dilution and elution buffer. Albumin was added to the sample in concentrations of 1–4 mg/ml.

3.2. Lateral flow driven by capillary forces

In dry paper, membrane and thin-layer chromatography wetting liquids flow spontaneously by capillarity. The liquid is wetting the walls of empty capillaries or pore spaces and the mean flow velocity ($<v>$) is depending on the surface tension (γ), viscosity (η) and contact angle of the liquid (θ), the radius of the capillary (r_c) and the distance from the liquid reservoir (X_f) [16] as shown in Eq. (1).

$$<v> = \frac{r_c \gamma \cos\theta}{4X_f \eta} \quad (1)$$

The buffer pad, the backed porous membrane and the absorbent sink shown in Fig. 1, all influence the flow in the device. The resulting rate of migration of substances in the membrane can be affected by the flow-rate in and between the different parts which build up the device and also by the degree of liquid saturation of the buffer pad and the absorbent sink. The flow-rate has been determined by bromophenol blue (BFB), which was found to migrate nicely together with the flow front.

The flow can in principle be driven by capillarity in the membrane, as well as in paper chromatography. When applying a wet buffer pad on a dry nitrocellulose membrane such a capillary flow is obtained as can be seen in Fig. 3(A). As expected from Eq. (1) the flow-rate decreases with an increase in migration distance.

On the other hand, when BFB was applied onto an already wetted membrane (with the flow driven by the capillarity of the absorbent sink) the zone was found to migrate at a rate which was nearly constant along the membrane. Use of an absorbent sink will thus ensure that the flow assumes a rate that for all practical purposes is constant a desired situation when the membrane is to be used for chromatography.

In Fig. 4 is shown the BFB migration rate (between the positions corresponding to 1 and 2 cm of migration along the membrane) for a nitrocellulose membrane with nominal pore sizes of 3 and 8 μm respectively, and with varying total lengths mounted with an absorbent sink (GB004). The BFB migration rate was about 2.6 times faster when using a membrane length of 3 cm instead of 7 cm. It can

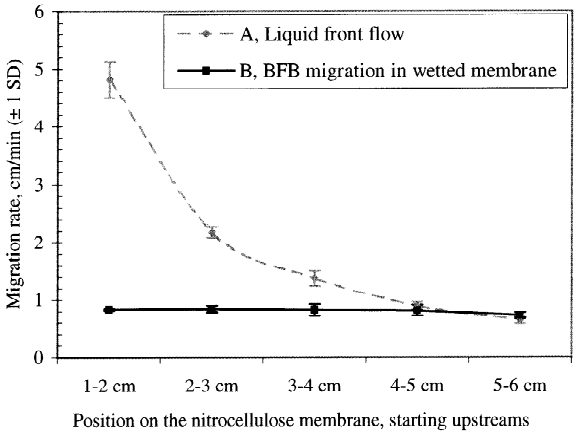

Fig. 3. Capillary driven flow in dry and wet membranes. A saturated buffer pad was applied on top of a nitrocellulose membrane with a nominal pore size of 8 μm (1×7 cm) containing an absorbent sink GB004 (1×3 cm) mounted at the downstream end. The rate of advancement of the liquid front along the membrane decreased rapidly as expected for a flow based on capillarity (A). After the membrane had been wetted all the way to the absorbent sink, an aliquot of bromophenol blue (BFB) solution was placed on the membrane and the migration rate of the BFB along the membrane was measured (B). In this case the flow-rate was constant along the membrane. This finding can be explained by the fact that the flow was driven by the capillary forces in the absorbent sink but the flow-rate was limited by the nitrocellulose membrane.

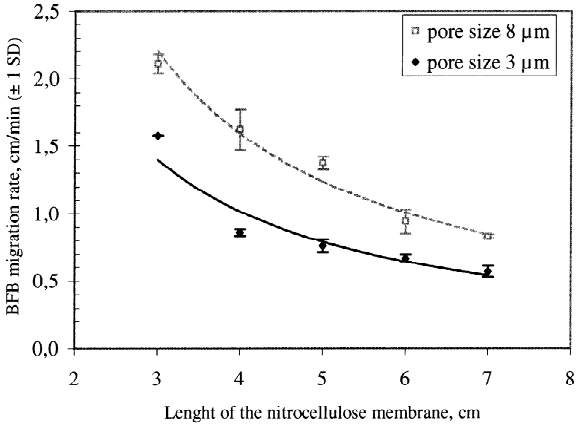

Fig. 4. Flow rate regulation. The BFB flow-rate measured at the 1–2 cm position is plotted as a function of strip length for pre-wetted nitrocellulose membranes of two porosities (3 and 8 μm). The flow-rate is seen to decrease when longer membranes or membranes with smaller pore sizes are being used.

also be seen that the 8 μm membrane gave a higher BFB migration rate than the 3 μm one (about 1.6 times higher). Thus it is possible to change the flow-rate by choosing different lengths and nominal pore sizes of the nitrocellulose membrane.

The influence of different absorbent sink materials on the flow-rate in the nitrocellulose membrane was tested and the result is shown in Table 1. Sink materials were chosen that showed large differences in front flow-rate, when measured at the position located between 1 and 2 cm along the direction of flow. These materials were tightly mounted on a pre-wetted 3 μm nitrocellulose membrane and the flow-rate for BFB (between the 1 and 2 cm locations) was measured. Table 1 shows that the high flow-rate possible in the sink was sharply retarded (>85%). This was probably due to the resistance of drawing liquid through the small pores of the membrane. Furthermore, the different sink materials gave distinct differences in the membrane BFB migration rate, although this rate was not a reflection of the front flow-rate in the sink. This implies that the flow can be regulated by the choice of sink material.

When comparing the rates at the beginning of the membrane (position between 1and 2 cm from the beginning) and at the end (position between 5 and 6 cm from the beginning) one observes a decrease in flow-rate that seems to be due to the type of absorbent sink. This flow-rate gradient was not seen when using the 8 μm membrane. Thus, both the surface properties of the materials and the pore size appear to influence the transport through the interface between the membrane and the absorbent sink.

The mean variation in migration rate, expressed as

Table 1
Properties of the absorbent sink

Absorbent sink	Front flow rate in abs sink cm/min	Flow rate in 3 μm membrane (BFB flow) cm/min (±1 SD)	Flow rate for pos 5–6 cm/ pos 1–2 cm %
Gelman 165	23	0.56 (±0.03)	55
Gelman 197	17	0.60 (±0.07)	70
GB004	11	0.64 (±0.05)	77
Whatman 1.5	10	0.68 (±0.03)	79
Whatman 17 Chr	4.5	0.72 (±0.03)	59

the coefficient of variation, was 3.9% and 6.5% respectively for the 3 and 8 μm membranes in this manually managed system using individual membranes. The highest obtained steady state flow-rate in this capillary flow driven systems was 2.1 cm/min for a 8 μm membrane.

3.3. Plate height in the non-substituted membrane

The zone broadening effect of the separation system affects its resolution [16]. Among factors which impact efficiency, the molecular diffusion gives rise to a random (Gaussian) spreading of the molecules. Another random event that can contribute to the zone broadening is the multipath dispersion. All molecules are not passing exactly the same way in the separation media, some travel shorter routes and other longer routes. The interaction of the molecules with both the stationary and the mobile phase is also giving rise to a spreading, the mass transfer dispersion. The cumulative spreading, $D_{T,}$ can be summarised as in Eq. (2) where D is the diffusion constant and D_{sp} is the contribution from the multipath and mass transfer dispersion.

$$D_T = D_{sp} + D \quad (2)$$

The time dependence for spreading of the molecules can be seen in Eq. (3) where σ^2 is the variance for the spreading, D_T is the total effective diffusion coefficient for the molecule, and t is the spreading time.

$$\sigma^2 = 2D_T t \quad (3)$$

The spreading along the separation path can be expressed by Eq. (4) where H, the plate height, is the symbol for the separation efficiency and X is the separation distance.

$$H = \frac{\sigma^2}{X} \quad (4)$$

In addition to the spreading effects caused by the matrix the plate height is also depending on the width of the applied sample zone in the direction of flow. For constant injection volumes this plate height contribution has a constant value regardless of flow-rate. It is, therefore, possible to correct for the zone broadening caused by the sample volume as can be seen in Eq. (5).

$$\sigma^2_{total} = \sigma^2_{column} + \sigma^2_{sample} \quad (5)$$

The local plate height can clearly vary along the separation path. In column separations there are as a rule no convenient methods to estimate the local plate height during the separation. By contrast, the membrane based thin-bed technology used in this study made it easy to follow the zone broadening process of coloured substances during the separation. By using BFB as the model substance and performing the separation in a flat-bed scanner it was possible to measure the zone broadening during the process as can be seen in Fig. 5.

Fig. 6 shows the measured parameters (±1 SD) for the migration of BFB in 7 cm long nitrocellulose membranes, beginning with the first measured peak

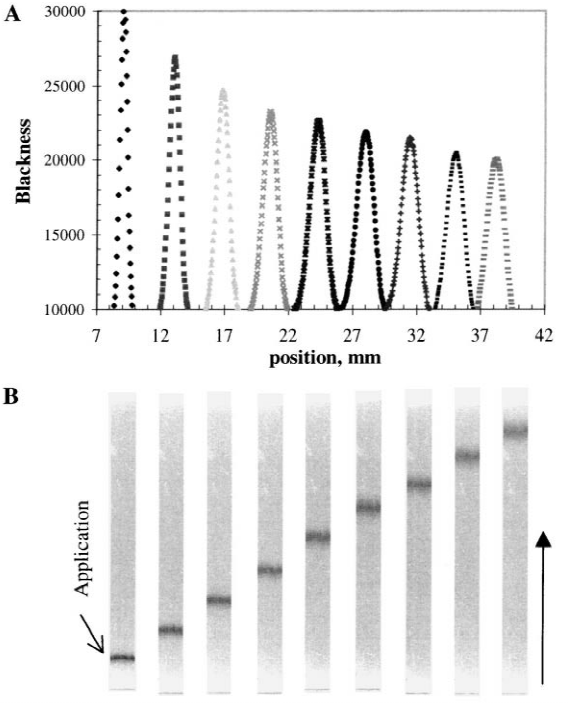

Fig. 5. Measurement of zone broadening by means of a flat-bed scanner. The migration of BFB in the nitrocellulose membrane is followed by placing the membrane in a scanner and repeatedly scanning the zone as it moves along the membrane. The scanned images, as seen in B, were digitised to yield the Gaussian distributed peaks seen in A.

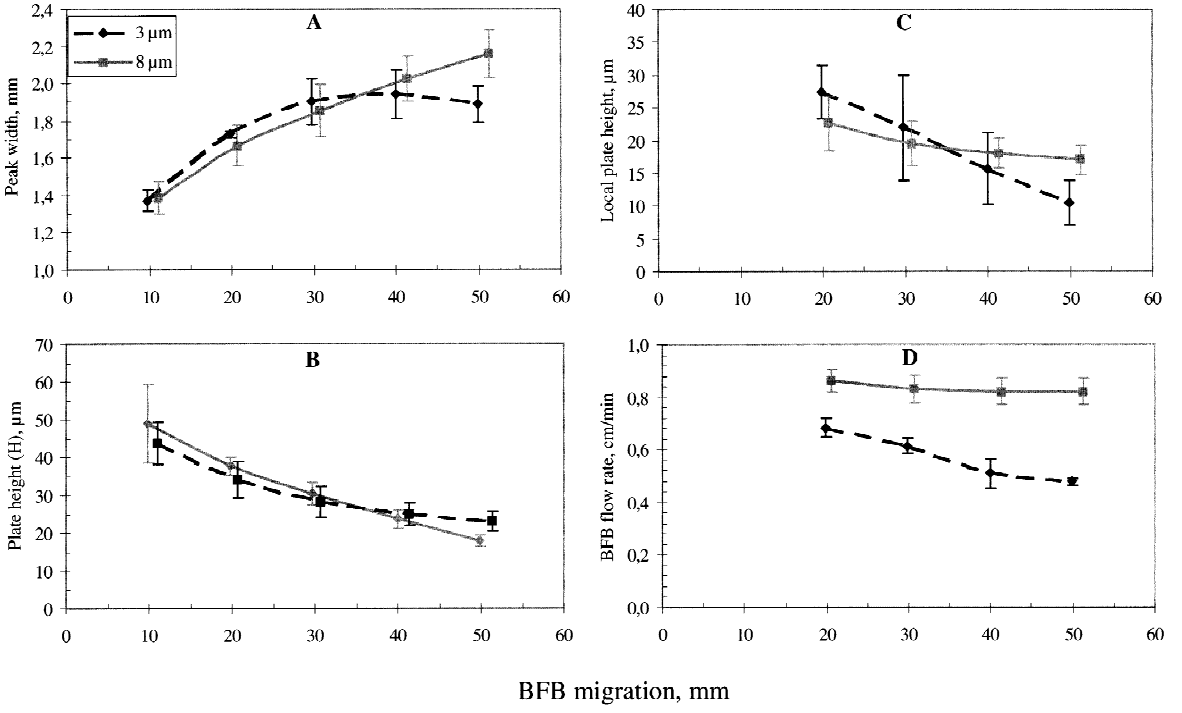

Fig. 6. Plate height estimation. The zone broadening during BFB migration was studied in membranes with pore sizes of 3 μm and 8 μm. In Fig. 6A the peak width at 0.607 of its height is plotted against the distance of migration and Fig. 6B shows the calculated plate height. Fig. 6C shows the local plate heights at 20, 30, 40 and 50 mm corrected by subtraction of the value measured at 10 mm and in 6D is plotted the BFB migration rate for these distances. The plate height decreases at longer migration distances, probably due to the progressively reduced influence of the sample volume applied. However, even after correction for sample volume, the local plate height decreases for the 3 μm membrane at longer migration distances, probably due to a flow-rate gradient.

width after 10 mm of migration. In Fig. 6A, a broadening of the peak width can be seen for the 8 μm membrane when BFB was migrating along the membrane. For the 3 μm membrane both a broadening and then a slight compression of the peak width was seen. When converted into plate height one can see in Fig. 6B that higher values are obtained in the beginning of the separation than at the end. The applied sample volume clearly affects this measure, as the constant σ^2_{sample} contributes proportionally more in the initial part of the separation, yet, even after this correction the local plate height for the 3 μm membrane reaches different values along the migration path, which seems to be a reflection of the zone compression as observed in Fig. 6A. In Fig. 6D the flow-rate is shown to slow down for the 3 μm membrane and, when simultaneously tested at two positions (between the 1–2 cm and 4–5 cm), it was found that the figure for the latter position was 80% of the first one. The flow into the absorbent sink was probably rate limiting, thus giving rise to the flow-rate gradient. This gradient, in turn, resulted in a compression of the BFB spreading zone. The effect of this phenomenon on the resolution is not clear but selection of a suitable membrane and sink should reduce the rate gradient.

After correcting for the applied sample volume the measured plate height was about 10 to 20 μm during migration from the 1 cm to the 5 cm position along the membrane. This value compares favourable with the plate height values reported for high-performance monolith chromatography. With the latter technique a plate height of 18 μm [5] was shown for thin glycidylmethacrylate–ethylenedimethacrylate membranes, which approaches the values for conventional HPLC columns packed with 5–7 μm porous particles. For a monolithic silica rod column, 9 μm in plate height was observed [17].

3.4. Introduction of charged groups on nitrocellulose membranes and measurement of the degree of substitution

Polyethylenimine (PEI), a highly branched positively charged polymer with the repeating unit C_2H_5N, is known to adsorb strongly to surfaces of materials such as sulfonated polyethylene, mica and silica [18,19] and, thereby, alter their charge. Below we report the use of this simple technique to modify nitrocellulose membrane.

The most commonly used production procedure for PEI gives primary, secondary and tertiary amino groups in the approximate ratio 1:2:1 with about one branch for every 3 to 3.5 nitrogen atoms [20]. PEI is considered to be non-charged at pH 10.8 and the degree of protonation does not exceed 75% at pH 2. It is available in a range of molecular weights (400–5 000 000 g/mol, expressed as light scattering derived mass average) with broad distribution. In this work we used a type with a molecular weight average of 750 000 g/mol.

The membrane bound PEI can not be characterised by elemental analysis since the nitrocellulose membrane itself contains about 11% nitrogen, and the small amounts of introduced PEI-amino nitrogen constitute less than 2% of the existing nitrogen content. The obtained degree of substitution of PEI was, therefore, measured by reacting both primary and secondary amino groups with sulfo-succinimidyl-3-(4-hydroxyphenyl) propionate (sulfo-SHPP) [21]. The introduced hydroxyphenyl group is capable of reducing Cu^{2+} to Cu^{+} in alkaline medium, a conversion that can be measured by incubation with 2,2′-biscinchoninic acid (BCA). BCA forms a Cu^{+}-chelate complex absorbing at 562 nm. Primary amino groups were determined by reacting SPDP [*N*-succinimidyl-3-(2-pyridyldithio) propionate] with the PEI membrane. The introduced 2-pyridyl disulfide groups were then reduced and the released pyridine-2-thione was measured by spectrophotometry. It was found that the PEI adsorption was relatively rapid. A maximum degree of substitution was reached after around 3 h (and 80% of the maximum figure after 0.5 h).

The dependence of the amount adsorbed on the concentration of PEI was examined by incubating a 3 μm nitrocellulose membrane during 3 h in solutions containing 0.03, 0.1, 0.3, 0.9 and 2.7% PEI. The adsorbed amounts of PEI-nitrogen were 9.7, 13.3, 16.6, 21.5 and 30 μmol/cm^3, respectively, when detected by the sulfo-SHPP reaction. All results were converted to total PEI-nitrogen by using the proportions 1:2:1 for the primary, secondary and tertiary amino groups. The results were about 17% lower when using the SPDP method which puts in question the validity of the mentioned proportions of primary, secondary and tertiary amino groups in the used PEI.

Compared to traditional ion-exchangers e.g. commercial ones such as DEAE Sepharose® and Mono Q® (Amersham Pharmacia Biotech AB, Sweden) which have 150–300 μmol of charged groups/cm^3 of packed gel, 3 μm anion-exchange nitrocellulose membranes prepared had very low degrees of substitution. However, our results show that membranes with this relatively low capacity (low density of charges) perform well in isocratic separation of the transferrin isoforms on an analytical level. In fact there are indications that even lower degrees of substitution might be preferable for certain applications.

The simple PEI based method for preparation of positively charged membranes seems reasonably reproducible and the obtained anion-exchange membranes showed good resolution. However, it might be advantageous to prepare derivatives with more commonly used groups like diethylaminoethyl or quaternary amino groups. This could e.g. be done through covalent attachment by activating the hydroxyl groups in the nitrocellulose or by using membranes with other easily substituted groups. For simple manufacturing, it would however be a clear advantage to use ligands conjugated to anchoring groups for quick spraying and drying on the membrane surface.

3.5. Protein binding capacity of anion-exchange nitrocellulose membrane

The capacity to bind human albumin (p*I* 4.8) was determined to 54 μg human albumin/cm^2 (4.3 mg/cm^3) for a 3 μm nitrocellulose membrane with a degree of substitution of 22 μmol PEI-N/cm^3. The bound albumin could be released to 90% by elution with a high ionic strength buffer. The capacity figure

can be compared to that reported for the Mono Q® matrix which is 65 mg bovine albumin/cm^3 [22]. Both the PEI-membrane and the Mono Q® matrix bind about 4 mol % albumin per mol of charged group. Thus, the binding capacity seems to be limited by the surface area rather than by the density of charges. A lower capacity to bind proteins may in fact be quite sufficient for analytical separations, if it is combined with a sensitive detection technique. It should be remembered however, that when performing separations of serum related components, albumin is often present at high concentration (about 40 mg/ml) compared to other proteins and will compete for the charged groups.

3.6. Evaluation of the anion-exchange nitrocellulose membrane

To perform an evaluation of the anion-exchange membrane without using a specific detection zone, as illustrated in Fig. 1, we selected to use ^{125}I-labelled proteins to measure them in the nanogram range directly on the separation membrane. This was done by detecting the appearance of, and quantifying the fraction of, the labelled proteins that had migrated for more than 1 cm from the application point. Observations for the different proteins are assembled in Figs. 7 and 8, which show times of migration, PEI substitution degrees and ionic strengths of the elution buffer obtained from individual strips that were used only once. A series of strips were prepared for each experimental set up and the different strips were loaded with one protein sample which was allowed to migrate for a fixed time period (2–10 min) in a buffer of a given composition.

Fig. 7 compares the separation of ^{125}I-asialotransferrin (p*I* 5.66), ^{125}I-tetrasialotransferrin (p*I* 5.32) and BSA (p*I* 4.8) when using different migration times for the individual strips. It is clear that asialotransferrin can be efficiently separated from tetrasialotransferrin and BSA since about 90% of the asialotransferrin had migrated more than 1 cm in 2 min whereas tetrasialotransferrin and BSA were not significantly present at the one cm position even after 10 min migration time.

Fig. 8 compares the performance of 4 membranes with varying degrees of substitution in terms of a 4 min separation of ^{125}I-asialotransferrin (p*I* 5.66),

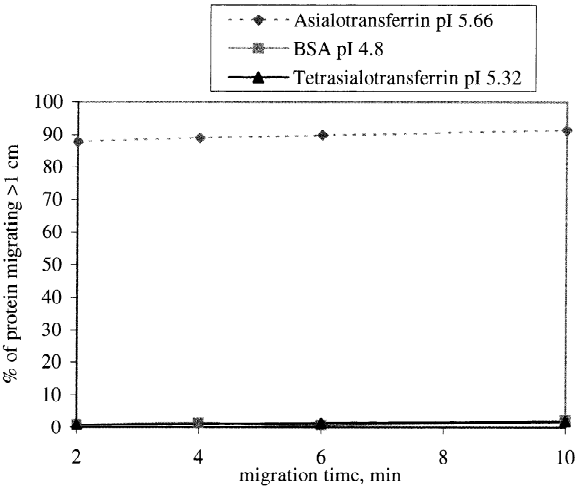

Fig. 7. Separation of ^{125}I-labelled proteins on anion-exchange nitrocellulose membranes, as a function of migration time. About 0.8 ng of the labelled proteins in 1 μl buffer containing 1 μg BSA were applied on a 5 μm membrane substituted with about 19 μmol PEI-N/cm^3. The elution was started by applying a buffer pad saturated with 20 m*M* bis-tris pH 6.30, 20 m*M* NaCl and 0.1% Tween 20. After different periods of time the flow was stopped by removing the buffer pad from the membrane. The membranes were then cut into pieces which radioisotope content was determined in a gamma counter. One membrane was used for each protein and migration time. The relative amount of label migrating more than 1 cm is plotted as a function of migration time.

^{125}I-disialotransferrin (p*I* 5.56), ^{125}I-tetrasialotransferrin (p*I* 5.32) and BSA (p*I* 4.8) using varying ionic strengths in the elution buffer. Every measurement in the figure represents an individually tested membrane device. As can be seen from the figure the separation system functions as expected of an anion-exchange matrix. Lower degrees of substitution or higher ionic strengths give lower amounts of the negatively charged proteins bound to the separation membrane. BSA (p*I* 4.8) did not migrate more than 1 cm, even for the lowest degree of substitution in combination with a high ionic strength (40 m*M* NaCl) elution buffer.

The efficiency of the separation of asialotransferrin (p*I* 5.66) and disialotransferrin (p*I* 5.56), with only a 0.1 unit difference in p*I*, was high. When testing the membrane with 17 μmol PEI-N/cm^3, using an elution buffer (pH 6.34) with 15 m*M* NaCl,

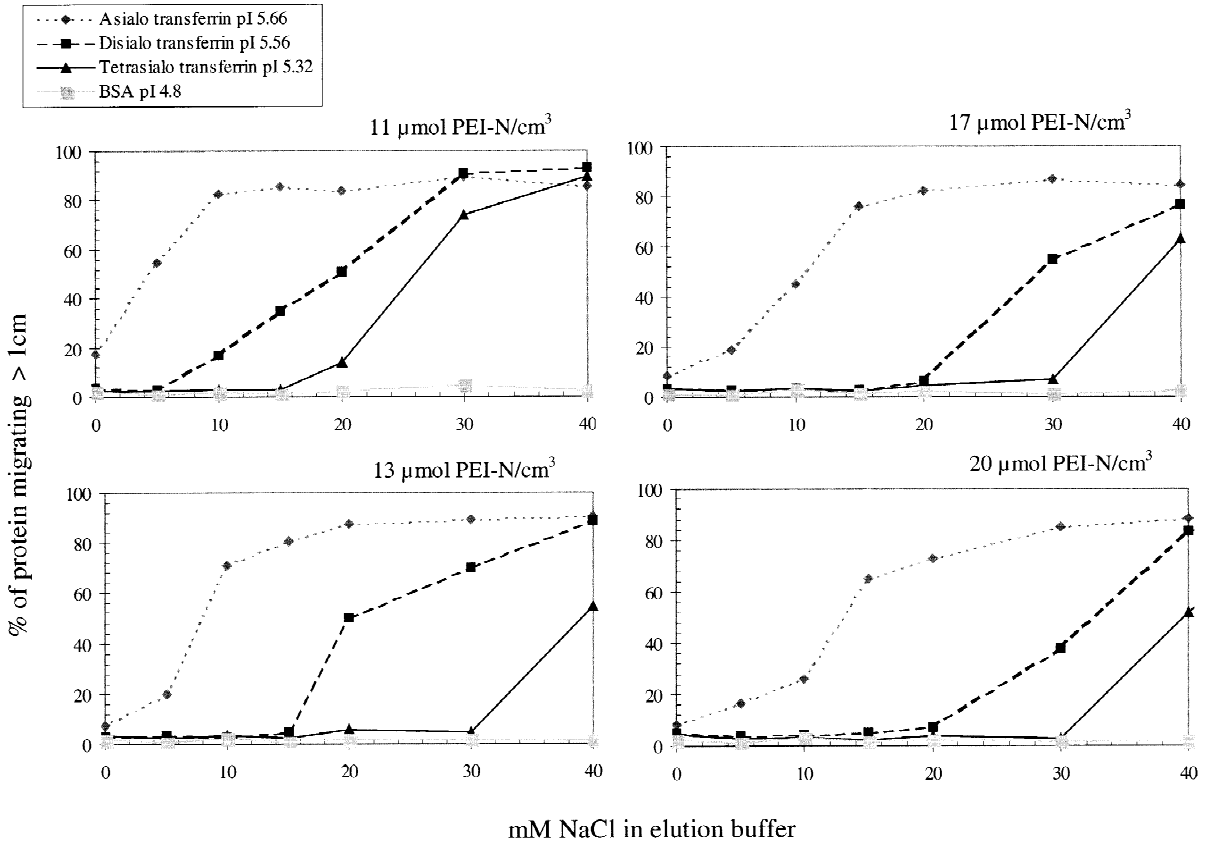

Fig. 8. Separation of ^{125}I-labelled proteins on anion-exchange nitrocellulose membranes with different PEI contents, as a function of the ionic strength of the elution buffer. Amounts of 0.55–3 ng of the labelled proteins in 1 μl of buffer containing 4 μg BSA were applied on the PEI membrane (one experiment for each protein and ionic strength). The elution was started by applying the buffer pad saturated with 20 m*M* bis-tris pH 6.33–6.38, 0.1% Tween 20 and 0–40 m*M* NaCl. After 4 min the flow was stopped by removing the buffer pad from the membrane and each sample detection was performed as in Fig. 7.

it appeared that 76% of asialotransferrin had migrated more than 1 cm while no significant amount of disialotransferrin could be detected at this position. The separation between disialotransferrin (p*I* 5.56) and tetrasialotransferrin (p*I* 5.32) was less efficient at the chosen pH. However, at lower pH the separation of these isoforms was better.

Variations in ionic strength, degree of substitution of the charged membrane, pH of the elution buffer, migration time and migration distance are some of the factors that govern the results of ion-exchange separations of this type and these factors can rapidly be tested out in the system using a parallel processing approach. Affinity chromatographic separation has also been tested with this technique. By using a cellulose matrix with covalently bound lectin from Sambucus nigra it was possible to separate asialotransferrin from tetrasialotransferrin in a few minutes [23].

3.7. Possibilities for detection of separated molecules

It should be possible to measure the separated molecules in, or downstream to, the separation zone

by detection methods generally used on nitrocellulose, such as staining of proteins, lipids and carbohydrates. Moreover, analysis with mass spectrometry is regularly performed on nitrocellulose membranes. However, due to the requirement of continuous capillarity it is difficult to perform UV detection on-line.

The features of the detection system determine the ability to measure analytes in complex biological liquids such as serum, where the target protein may represent a minor fraction (10^{-12}) of the total protein content. Low concentrations of target, in the femtomolar (10^{-15} *M*) range, and small sample volumes require detection systems of high sensitivity. It is common to use immunoassay based detection methods since they typically have both the sensitivity and specificity required.

As an example, IgE has been measured down to 0.13 p*M* in 10 min by means of a quantitative immunochromatographic test procedure with carbon black as a label [9]. Besides high sensitivity, it is important for the detection equipment to have high optical resolution, which together with the chromatographic resolution sets the ability to detect narrow peaks. The flatbed scanner used here could measure 24 pixels/mm and gave acceptable precision for a measuring area of 0.2 mm^2 [9].

3.8. Potential applications

The ability to rapidly measure protein isoforms has many potential applications. A relevant example is illustrated by the testing of alcohol abuse that can be performed by determining the carbohydrate-deficient transferrin isoform concentration [24]. The on-line combination of anion-exchange and immunochromatographic detection, possible to use in the point-of-care situation, has recently been presented as the first example of the MAIIA (Membrane Assisted Isoform ImmunoAssay) technology [13] for the determination of these less negatively charged isoforms. Doping testing of recombinant erythropoietin (EPO) in sport games [25] can probably also be performed by the described technique, since recombinant EPO has a different charge compared to endogenous EPO due to the glycosylation pattern. So far, no rapid and simple test has been developed that can measure this difference.

4. Conclusions

The use of ion-exchange and affinity separation modes with this kind of simple separation tools, showing plate heights of 10–20 μm, seems to compare well with other analytical scale separation techniques performed with expensive and complicated equipment. With the present technique a range of membranes with different substitution degrees, pore sizes, separation lengths can be prepared. In addition, membranes with different property may be mounted in sequence to produce gradients of charge and affinity to further enhance the separation power.

In addition to the reported ion-exchange separation with the ability to separate proteins differing with 0.1 p*I* units in some minutes, the same separation concept has also been showing good results in an affinity chromatography mode using lectins as specific adsorbents.

The concept of performing chromatography and detection on the same device without any need for external regulation equipment is indeed thrilling. The possibility offered by the flat and thin porous film to guide flow in any and through any separation environment, any temperature zone as well as any reaction and detection zones has considerable analytical appeal.

Tracks can be printed in the membrane, which allow the running of multiple columns in parallel on a small surface area [26]. The convective flow in the membrane, together with the short diffusion distances to immobilised reactive groups, enhances the reaction kinetics. Another advantage is that the low cost of the material makes it possible to use the separation device as a disposable unit, thereby avoiding regeneration problems.

While the quantification of protein isoforms discussed above constitutes a difficult separation problem of clinical relevance, the analytical process described here should also be of general interest as a rapid and high resolution separation technique for proteins and nucleic acids both in research and for monitoring industrial processes.

Acknowledgements

The authors wish to thank Anders Lundequist for technical assistance. The authors also wish to thank Doctor Karin Caldwell for valuable discussions and linguistic revision of the manuscript.

References

[1] J. Thömmes, M.-R. Kula, Biotechnol. Prog. 11 (1995) 357.
[2] T.B. Tennikova, F. Svec, J. Chromatogr. 646 (1993) 279.
[3] T.B. Tennikova, R. Freitag, J. High Resol. Chromatogr. 23 (2000) 27.
[4] K.-G. Briefs, M.-R. Kula, Chem. Eng. Sci. 47 (1992) 141.
[5] A. Podgornik, M. Barut, J. Jancar, A. Štrancar, J. Chromatogr. A 848 (1999) 51.
[6] A. Podgornik, M. Barut, J. Jancar, A. Štrancar, T. Tennikova, Anal. Chem. 71 (1999) 2986.
[7] T.I. Pristoupil, Chromatog. Rev. 12 (1970) 109.
[8] C.P. Price, G.H.G. Thorpe, J. Hall, R.A. Bunce, in: C.P. Price, D.J. Newman (Eds.), Principles and Practise of Immunoassay, 2nd ed., Macmillan reference Ltd, London, 1997, p. 580.
[9] M. Lönnberg, J. Carlsson, Anal. Biochem. 293 (2001) 224.
[10] G.A. Turner, Clin. Chim. Acta 208 (1992) 149.
[11] A. Varki, Glycobiology 3 (1993) 97.
[12] G. Durand, N. Seta, Clin. Chem. 46 (6) (2000) 795.
[13] M. Lönnberg, J. Carlsson, J. Immunol. Meth. 246 (2000) 25.
[14] A.S. Said, in: W. Bertsch, W.G. Jennings, R.E. Kaiser (Eds.), Theory and Mathematics of Chromatography in Chromatographic Methods, Hüthig, Heidelberg, 1981, p. 126, Chapter 3.
[15] J. Carlsson, H. Drevin, R. Axén, Biochem. J. 173 (1978) 723.
[16] J.C. Giddings, Unified Separation Science, Wiley, New York, 1991.
[17] K. Cabrera, D. Lubda, H.-M. Eggenweiler, H. Minakuchi, K. Nakanishi, J. High Resol. Chromatogr. 23 (2000) 93.
[18] N. Larsson, P. Stenius, J.C. Eriksson, R. Maripuu, B. Lindberg, J. Colloid Interface Sci. 90 (1982) 127.
[19] P.M. Claesson, E. Blomberg, O. Paulson, M. Malmsten, Colloids Surfaces A: Physicochem. Eng. Aspects 112 (1996) 131.
[20] D. Horn, in: E.J. Goethals (Ed.), Polyethylenimine — Physicochemical Properties and Applications in Polymeric Amines and Ammonium Salts, Pergamon Press, Oxford, 1979, p. 333.
[22] E. Karlsson, L. Rydén, J. Brewer, in: J.-C. Jansson, L. Rydén (Eds.), Protein Purification, 2nd ed., Wiley-Liss, New York, 1998, p. 145, part II-4.
[21] S.E. Kakabakos, P.E. Tyllianakis, G.P. Evangelatos, D.S. Ithakissios, Biomaterials 15 (4) (1994) 289.
[23] J. Carlsson, M. Lönnberg (1999) In. Pat. appl. PCT/SE99/00722.
[24] O. Mårtensson, A. Härlin, R. Brandt, K. Seppä, P. Sillanaukee, Alcohol Clin. Exp. Res. 21 (1997) 1710.
[25] L. Wide, C. Bengtsson, B. Berglund, B. Ekblom, Med. Sci. Sports Exerc. 27 (1995) 1569.
[26] R. Bunce, G. Thorpe, L. Keen, Anal. Chim. Acta 249 (1991) 263.