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Journal of Chromatography B, 713 (1998) 301–311

JOURNAL OF CHROMATOGRAPHY B

Protein analysis by membrane preconcentration–capillary electrophoresis: systematic evaluation of parameters affecting preconcentration and separation

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Received 3 February 1998; received in revised form 24 April 1998; accepted 27 April 1998

Abstract

Fast and efficient analysis of proteins in physiological fluids is of great interest to researchers and clinicians alike. Capillary electrophoresis (CE) has proven to be a potentially valuable tool for the separation of proteins in specimens. However, a generally acknowledged drawback of this technique is the limited sample volumes which can be loaded onto the CE capillary which results in a poor concentration limit of detection. In addition, matrix components in samples may also interfere with separation and detection of analytes. Membrane preconcentration–CE (mPC–CE) has proved to be effective in overcoming these problems. In this report, we describe the systematic evaluation of parameters affecting on-line preconcentration/clean-up and separation of protein mixtures by mPC–CE. Method development was carried out with a standard mixture of proteins (lysozyme, myoglobin, carbonic anhydrase, and human serum albumin). First, using MALDI-TOF-MS, membrane materials with cation-exchange (R-SO_3H) or hydrophobic (C_2, C_8, C_{18}, SDB) characteristics were evaluated for their potential to retain proteins in mPC cartridges. Hydrophobic membranes were found most suitable for this application. Next, all mPC–CE analysis of protein samples were performed in polybrene coated capillaries and parameters affecting sample loading, washing and elution, such as the composition and volume of the elution solvent were investigated. Furthermore, to achieve optimal mPC–CE performance for the separation of protein mixtures parameters affecting postelution focusing and electrophoresis, including the composition of the background electrolyte and a trailing stacking buffer were varied. Optimal conditions for mPC–CE analysis of proteins using a C_2 impregnated membrane preconcentration (mPC) cartridge were achieved with a background electrolyte of 5% acetic acid and 2 m*M* ammonium acetate, 60 nl of 80% acetonitrile in H_2O as an elution solvent, and 60 nl of 0.5% ammonium hydroxide as a trailing stacking buffer. The developed method was used successfully to separate proteins in aqueous humor, which contains numerous proteins in a complex matrix of salts.

Keywords: Preconcentration; Proteins

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0378-4347/98/$19.00
PII: S0378-4347(98)00209-6

1. Introduction

The emergence of proteomic research has recently heightened interest in the analysis of complex mixtures of proteins. However, analysis of these important biopolymers remains a challenge. First, there are no known methods to amplify the concentration of these molecules. Secondly, subtle changes in physical or chemical environments can readily promote degradation, denaturation and aggregation, which often renders these analytes insoluble. Small sample consumption and highly efficient analyte separations in aqueous solution have rendered CE a very attractive method for protein analysis. Indeed, free solution CE (CZE) has been successfully applied to a number of protein separations [1–4]. Using this technique analytes are separated in narrow bore capillaries under the influence of an electric field by a mechanism that is based (to a first approximation) upon their charge-to-mass ratio [5]. Unlike slab gels, separations in such thin-walled capillaries allow efficient heat dissipation and the ability to apply very high electric field strengths ($>$500 V/cm). This often results in exceptionally high separation efficiencies, good analyte resolution and short analysis times. However, the inner surface of fused-silica capillaries is not chemically inert and analytes may be adsorbed by a number of mechanisms including hydrophobic and electrostatic ion-pairing interactions [6]. In addition, the formation of H-bonds between proteins and capillary wall can lead to further losses as well as severe peak broadening and changes in the EOF resulting in irreproducibility of separations [7,8].

Major efforts have been directed towards the elimination of unwanted protein–surface interactions by shielding the capillary surface, or inversion of the negative surface charge of the capillary wall [7,8]. Approaches include the use of capillary coatings of polymers such as polyacrylamide, polyethylene glycol, polyethylene imine or C_{18} moieties covalently attached to the capillary wall [9–12]. Static coatings (e.g. polybrene) adsorbed onto the capillary surface prior to separations and dynamic coatings with materials contained in the separation buffer (e.g. methyl cellulose) can be used to minimize or effectively negate protein adsorption [13,14]. We have also investigated various capillary surface modifications for protein separations at low pH and found selected neutral and positively charged coatings advantageous. Indeed, for our applications, polybrene coated capillaries proved to be particularly useful [15].

A reported significant advantage of CE over other contemporary techniques (e.g. HPLC) is the small sample volume required for analysis (1–40 nl). However, the limited sample loading capabilities of CE is also a major disadvantage, resulting in poor concentration limits of detection [16]. In this regard, a number of different approaches have been developed to overcome the limited sample volume without loss of CE performance [16,17]. These include analyte stacking, where analytes present in a low conductivity solvent focus in narrow bands upon application of the high voltage, resulting in a substantially increased concentration [18,19]. The discontinuous buffer regime of transient isotachophoresis (tITP) has also been developed for on-column trace enrichment of components in low ionic strength samples [20–22]. Other techniques that were developed specifically for the analysis of dilute protein solutions include coupled capillary isoelectric focusing with CZE (cIEF–CZE) [23] and protein collection onto a moving membrane following separation in wide bore (100 μm I.D.) CZE capillaries [24]. In addition, chromatographic techniques have been described for on-line sample preconcentration prior to analysis by CE. These have included the use of an analyte concentrator, which contains an affinity capture material for the selective isolation and preconcentration of a specific analyte [25–27]. In other studies, devices containing hydrophobic HPLC phases in miniaturized packed precolumns and coupled capillary isotachophoresis (cITP) with CZE have all been reported for use on-line with the CE capillary [28–36]. More recently, an on-line membrane preconcentration approach has been developed in our group and extensively applied to a variety of analytical problems involving low concentrations of small molecules and peptides in complex matrices [37–42]. This technique, termed membrane preconcentration–CE (mPC–CE), utilizes commercially available membranes impregnated with conventional HPLC stationary phases. It is an on-line process that enables the application of large sample volumes to affect both sample preconcentration and clean-up. In addition, potential analyte losses due to extensive

off-line sample handling are minimized. Sample volumes in excess of 200 μl have been analyzed by this technique, and low attomole concentration sensitivity has been accomplished [37,42].

In the present study, we describe the optimization of preconcentration and separation parameters for the analysis of protein mixtures by mPC–CE. To our knowledge, this is the first systematic study in which the complex characteristics of these macromolecules are considered in conjunction with low level protein analysis by mPC–CE. Parameters such as the type of membrane, composition and volume of elution solvent, composition of the background electrolyte (BGE) and influence exerted by variations in concentrations and volume of a trailing stacking buffer (TSB) have been examined by the analysis of a standard mixture of proteins. We also report the application of an optimized mPC–CE methodology to the analysis of proteins in physiological fluids, and in this study we show the analysis of human aqueous humor.

2. Materials and methods

2.1. Materials

Glacial acetic acid, trifluoroacetic acid (TFA), lysozyme, myoglobin, carbonic anhydrase and human serum albumin (HSA) were purchased from Sigma (St. Louis, MO, USA). Ammonium acetate (NH_4OAc), polybrene, ethylene glycol and sinnapinic acid were obtained from Aldrich (Milwaukee, WI, USA) all with a purity of ≥99%. Deionized water, ethanol and acetonitrile were purchased from Burdick & Jackson (Muskegon, MI, USA). Methanol was obtained from EM Science (Gibbstown, NJ, USA). Aqueous humor samples of ≤100 μl each were obtained by paracentesis during cataract surgery. Bare fused-silica capillaries were purchased in bulk from Polymicro Technologies (Phoenix, AZ, USA). Styrene divinyl benzene (SDB), C_8, C_{18} impregnated membranes (3M Empore™) were purchased from Varian (Harbor City, CA, USA). C_2, cation-exchange and cation-exchange/SDB impregnated membranes were obtained as a gift from David Wells of 3M (St. Paul, MN, USA).

2.2. mPC–CE conditions

All mPC–CE studies were performed with a Beckman P/ACE system 5000 or 2000 series instrument (Fullerton, CA, USA). Separations were monitored at a wavelength of 214 nm. If not otherwise indicated the capillary length was 57 cm (50 cm effective length). The BGE was composed of an aqueous solution of acetic acid (1–10%, v/v) and 2 m*M* NH_4OAc, and the applied separation voltage was −15 kV. Protein stock solutions of 80 pmol/μl and dilutions thereof at 20 pmol/μl (for MALDI-TOF-MS experiments) and 0.5 pmol/μl (for mPC–CE studies) were prepared in water. Aqueous humor (8–10 μl) was placed in microvials for direct injection into the capillary or loading onto the membrane.

The coating procedure for polybrene is described elsewhere [14]. Briefly, capillaries were flushed with 0.5 *M* potassium methoxide solution and water for 30 min each. Then a solution containing 5% polybrene and 2% ethyleneglycol was pushed through the capillary for 5 min and allowed to remain in the lumen for another 5 min. The last two steps involving the polybrene solution were repeated three times before the capillary was rinsed with methanol for 10 min and BGE for 10 min. To condition the coating a voltage of −15 kV was applied across the capillary for 10 min.

mPC–CE cartridges were constructed as previously described [37]. Briefly, a small membrane plug was inserted into a PTFE tube. Two capillary pieces were placed from both ends into the PTFE tube allowing for some space between the membrane and the capillary ends to maintain electrical conductivity. The assembly was next attached to the inlet side of the separation capillary via a small piece of polyethylene (PE) tubing. Prior to the separation, the membrane was activated with methanol and a 1% solution of HOAc (5 μl each). The flow-rate of the BGE through the assembled mPC–CE capillary at an applied pressure of 20 p.s.i. (1 p.s.i.=6894.76 Pa) was determined. Optimal performance was achieved when the BGE flow-rate was between 1.0 and 1.2 μl/min.

mPC–CE analyses were carried out as follows. A predetermined sample volume was loaded onto the membrane which was washed with BGE, and the

proteins were eluted with a solution containing an appropriate organic solvent. For postelution focusing a dilute solution of NH_4OH was used. To prevent continued interaction of the eluate with the membrane a zone of BGE (240 nl) was injected into the capillary before application of the separating voltage.

2.3. Evaluation of membranes by MALDI-TOF-MS

MALDI-TOF-MS analysis was carried out using a Bruker BiFlex 1 (Billerica, MA, USA). A nitrogen laser with an output of 337.1 nm was used for these studies. An accelerating voltage of 25 kV was used throughout, and the instrument was externally calibrated using cytochrome *c*.

Preconcentration cartridges were similar to those used in mPC–CE experiments, each contained a small piece of the test membrane. To determine the type best suited for protein preconcentration six different membranes (see Section 2.1) were evaluated. The general testing procedure was carried out off-line from the mass spectrometer and was comprised of conditioning steps (2×5 μl) followed by loading (2 μl), washing (10 μl) and elution steps (2×1 μl). Membranes containing sulfonated cation-exchange material or a mixed phase of cation exchanger and styrene divinyl benzene (SDB) were activated with 1 *M* NH_4OAc and 1 *M* NH_4OAc in 50% methanol, respectively and washed with water. The other four membranes containing particles of reversed-phase material such as SDB and silica based C_2, C_8 and C_{18} embedded in a carrier of polytetrafluorethylene (PTFE) were activated with consecutive washes of methanol and a 1% solution of acetic acid. Protein elution was facilitated with 80% methanol (2×1 μl). Specific fractions eluting from each test cartridge were deposited on a preformed matrix of sinnapinic acid. Upon drying, another 1 μl of matrix solution was applied to the sample spot, in addition to 1 μl of 5% TFA. The resultant sample was air dried prior to analysis.

In these experiments, while the total protein loading capacity of the membranes was estimated to be ~10 pmol, current limitations of our MALDI-TOF-MS instrumentation led us to consciously overload the test membranes. Thus, all four of proteins of the standard mixture were detected in the loading and washing fractions of all membranes. However, this approach provided us with a means to rapidly reject those membranes that exhibited no protein retention.

3. Results and discussion

Previously, we have shown that a polybrene coated capillary was most appropriate for the separation of a physiologically derived protein mixture by CE [15]. Therefore, this was the coating of choice for the development of mPC–CE conditions to separate the components of aqueous humor. However, it is our experience that the separation efficiency of polybrene coated capillaries varies over the course of multiple consecutive separations as well as with of the age of the fused-silica capillary [15]. Therefore, prior to use and during a series of separations we routinely evaluate capillary performance with a standard mixture of proteins. Using the mixture of proteins listed in Table 1, a high quality polybrene coated capillary would typically exhibit three resolved UV responses and high separation efficiency (data not shown). Carbonic anhydrase and myoglobin were detected with the shortest migration times, and as the electrophoretic mobilities of these proteins were very similar, they were detected as one peak. Resolution of these proteins was, therefore, a

Table 1
Characteristics of proteins used in standard mixtures[a]

Protein	M_r	p*I*	Hydrophobicity (Bull & Breese index) [43]
Lysozyme	14 300	11.3	1140
Myoglobin	16 900	6.9, 7.35	−2000
Carbonic anhydrase	29 000	6.0	−45 667
HSA	66 400	5.4	−15 260

[a] These proteins were dissolved in water and further diluted with a solution of 1% aqueous acetic acid to yield a final concentration of 0.5 μ*M* of each (20 μ*M* of each for MALDI-TOF-MS experiments).

good indication of not only the quality of the capillary coating processes but also separation performance in subsequent mPC–CE experiments. Throughout these experiments, since no coating agent was added to the BGE, deterioration of the polybrene coated capillaries was observed after ~10–20 analyses. This was easily detected by the loss of separation efficiency caused by proteins adsorbing to the uncoated capillary. Recoating the capillary with polybrene usually recovered performance, and each capillary could be recoated ~10 times before a new piece of fused-silica capillary was required.

3.1. Evaluation of membranes for use in mPC cartridges

Impregnated membranes containing a variety of HPLC stationary phases were available to us in these studies. These included three membranes that were commercially available (SDB, C_8, and C_{18}) and three experimental membranes (C_2, a cation-exchanger, and a mixed phase cation-exchanger/SDB). The suitability of each membrane for retention of proteins was evaluated using MALDI-TOF-MS analysis of various fractions (break through, washings and elution) of a mixture of standard proteins (see Table 1). These proteins exemplify a wide range of characteristics, and model those potentially present in specimens of ocular fluids. The four membranes that passed this test contained reversed-phase material such as the polymeric SDB or silica based C_2, C_8 and C_{18} particles embedded in a carrier membrane of PTFE. However, based on chromatographic first principles for the analysis of proteins we elected to fully optimize protein analysis by mPC–CE using only the silica based C_2 membrane and this process is described below.

3.2. Optimization of elution solvent composition and volume

Previously, we have shown that the volume of eluate used to elute analytes from the membrane affects not only analyte recovery from the membrane but also the quality of the separation [37]. In the present case, reducing the volume of elution solvent to 30 nl led to no recovery of the test proteins (Table 1) from the C_2 membrane. In contrast, relatively large volumes of this solution ($\geq$120 nl) substantially decreased the electrophoretic separation such that HSA and lysozyme comigrated (data not shown). Therefore, a compromise must be made in which the volume of elution solvent is kept as low as possible while using a sufficient amount to ensure efficient analyte recovery [37]. In these studies, while 60 nl of an elution solvent comprised of 80% acetonitrile in water did not provide as optimal protein recoveries as we would have desired, these conditions did enable reasonable mPC–CE separation performance. Recovery of the standard was often $\leq$25% and analyte carryover was a significant problem. However, analysis of biologically derived protein mixtures (such as aqueous humor) yielded much improved protein recovery ($>$90%), suggesting that the sample matrix also plays a significant role in membrane performance.

The effects of different organic solvents (methanol, ethanol, and acetonitrile) on protein recovery from the C_2 membrane were next investigated. The results of these studies (summarized in Table 2) indicated that a solution of 80% acetonitrile in water provided the best recovery of protein from the C_2 membrane. Indeed, this solvent yielded approximately 3.4 times more signal for HSA than a methanolic solution of the same concentration in water (Table 2). Further evaluation of the effects of other potential elution solvent modifiers, such as acetic acid and TFA, upon mPC–CE performance were also conducted. From macro-scale studies, proteins in the standard mixture (80 pmol/μl of each in 150 μl solvent) tended to be denatured and precipitated in solvents containing $\geq$40% acetonitrile. However, addition of 1% acetic acid or 0.02% TFA aided in their dissolution so that up to 80% acetonitrile could be added to the protein solution without the formation of a precipitate. In mPC–CE studies, the amount of acetonitrile was lowered stepwise from 80% to 40% acetonitrile. With decreasing organic content of the elution solvent incomplete recovery of the proteins from the membrane was observed (see Table 2). Using 50% acetonitrile, carbonic anhydrase and myoglobin were not recovered from the membrane at all, and an elution solvent containing 40% acetonitrile enabled the recovery of only one of the proteins of the standard mixture (see Table 2). Therefore, an elution solvent of 80% acetonitrile in water was used

Table 2
Summary of method development studies for optimization of protein separation by mPC–CE

Concentration of TSB	Volume of TSB	Composition of elution solvent	Volume of elution solvent (nl)	Average peak width (min)	Separation profile	Analysis time (min)	Comments
N/A	N/A	80% MeOH in H_2O	60	~3	Carbonic anhydrase and myoglobin comigrated	~25	Broad diffuse peaks
N/A	N/A	80% EtOH in H_2O	60	~3	Carbonic anhydrase and myoglobin comigrated	~25	Broad diffuse peaks
N/A	N/A	80% ACN in H_2O	60	~1	All proteins were partially resolved	~20	Recovery was ~3.4× more than MeOH elution solvent
N/A	N/A	50% ACN in H_2O	60	~1	Only two proteins were detected	~20	Recovery was poor only HSA and lysozyme detected
N/A	N/A	40% ACN in H_2O	60	~1	Only one protein was detected	~20	Recovery was poor only lysozyme detected
0.1% NH_4OH in H_2O	60	80% ACN in H_2O	60	~1	All proteins were partially resolved	~20	Peak fronting was observed
0.5% NH_4OH in H_2O	60	80% ACN in H_2O	60	~0.5	All proteins were partially resolved	~20	Optimal performance, also see Fig. 1
1.0% NH_4OH in H_2O	60	80% ACN in H_2O	60	~0.5	All proteins were partially resolved	~20	Separation not as good as immediately above
5.0% NH_4OH in H_2O	60	80% ACN in H_2O	60	~0.2	All proteins comigrated	~10	All components migrated in one narrow zone

General conditions include the use of a polybrene coated capillary [57 cm (50 cm effective length)×50 μm I.D.]. Component detection was by UV at 214 nm. Separation potential was −15 kV. Injection volume was 1 μl of a solution containing carbonic anhydrase, myoglobin, human serum albumin and lysozyme (all at a concentration of 0.5 pmol/μl). A C_2 membrane preconcentration cartridge and a BGE consisting of 2 m*M* NH_4OAc in 1% aqueous acetic acid were used throughout.
NH_4OAc=ammonium acetate, NH_4OH=ammonium hydroxide, MeOH=methanol, EtOH=ethanol, ACN=acetonitrile, N/A=not applicable.

in all subsequent studies. We also determined that acidifying this elution solvent with acetic acid was not necessary. Typically, no significant quantitative or qualitative differences were detected in mPC–CE electropherograms for elution solvents comprised of 80% acetonitrile in water, or 80% acetonitrile modified with 1% acetic acid in water (data not shown). Indeed, on occasion the addition of acetic acid to the elution solvent significantly reduced the recovery of HSA. We rationalize this result by consideration of the construction of the mPC–CE cartridge. As these devices are assembled, dead volumes are deliberately introduced to prevent crushing the membrane, and cartridge interference with electrophoretic processes. Obviously, as solvents flow through such gaps in the mPC-cartridge turbulence and solvent mixing will occur. Therefore, while the elution solvent is 80% acetonitrile in water, there is likely to be different zones of changing elution solvent composition. For example, the front edge of the elution solvent will be diluted with BGE. Thus, indirectly the elution solvent will be modified with acetic acid, and this is likely to aid the dissolution of proteins as these analytes are eluted from the C_2 membrane. In other experiments, addition of 0.02% TFA to the elution solvent only had detrimental effects on the electrophoretic separation of proteins (data not shown). We attribute this latter result to the fact the TFA (an excellent ion-pairing agent) temporarily deactivated the positively charged capillary coating.

3.3. Optimization of BGE composition and protein focusing strategies

All mPC–CE electropherograms described above were characterized by a relatively low separation efficiency. We attempted to improve this situation by increasing the ionic strength of the BGE to prevent analyte–analyte and analyte–wall interactions [2]. As these studies were a prelude to on-line coupling with a mass spectrometer, our focus was to develop a volatile BGE. This limited us to using ammonium salts, and volatile organic acids. For protein analysis in a polybrene coated capillary, we have found that a BGE composed of ammonium acetate and acetic acid is quite useful. However, due to the low concentration of the complimentary base, such a BGE does not have any buffering capacity and the ammonium acetate is added for the sole purpose to maintain a minimum conductivity of this solution. As expected, increasing the ammonium acetate concentration in the BGE from 2 m*M* to 10 m*M* resulted in the detection of sharper analyte peaks, but at this increased ionic strength, protein resolution was lost (data not shown). Therefore, in subsequent experiments an ammonium acetate concentration of 2 m*M* was maintained. In addition, the effect of increasing the concentration of acetic acid in the BGE from 1% to 10% (v/v) was also examined. Here, analyte resolution was improved as the concentration of acetic acid in the BGE was increased. However, such improved performance also resulted in extended analysis times (data not shown). This was primarily attributed to an increased BGE viscosity with increasing acid concentrations. Ultimately, a BGE comprised of 2 m*M* ammonium acetate in 5% aqueous acetic acid was found optimal with respect to analyte resolution and analysis time.

An alternative method of improving analyte resolution and separation efficiency is the use of a stacking or focusing strategy at the start of electrophoresis to effect analyte preconcentration and zone focusing [44]. Previously, we have shown that it is important to use tITP in conjunction with mPC–CE for the analysis of peptide mixtures [37–39]. Typically, we have found that a tITP strategy in which analytes are eluted between an acidic leading stacking buffer (LSB) and a basic trailing stacking buffer (TSB) is optimal for peptide separation by mPC–CE [37–39,45]. In a systematic study, we reported that the mechanism of analyte focusing was quite complex, and was a composite of tITP, cIEF and analyte stacking [45]. We believed that this strategy was also appropriate to focus proteins that are separated by mPC–CE. In these experiments, the acidic BGE was also used as the LSB, and we focused our efforts upon optimization of the volume and concentration of a TSB of ammonium hydroxide in water. Both of these parameters were found to be of paramount importance, and directly affected the quality of the separation. Increasing concentrations of base from 0.1% to 5% in water tended to improve protein focusing (see Table 2). However, beyond an optimum concentration (>1% ammonium hydroxide in water) the resolution of the analytes was lost to the extent that all proteins comigrated (Table 2). A

similar effect was also observed on increasing the volume and therefore the zone length of the TSB. As the TSB zone length was increased the effect of the transient cIEF step also appeared to have more influence in protein separation. As a result the step pH gradient that forms within the capillary tends to take a longer time to disperse. While this led to improved separation efficiency, such gain was counteracted by a loss of protein resolution (data not shown). In these studies, 60 nl of a TSB comprised of 0.5% ammonium hydroxide in water was found to be optimal.

Following the introduction of TSB into the capillary, a further volume of BGE is used to move the eluted analytes away from the membrane. Since analytes can move toward either the anode or cathode at the start of electrophoresis, this zone of BGE is used to prevent readsorption of protein on the membrane as voltage is applied across the mPC–CE capillary. Throughout these studies, a volume of 240 nl of BGE was introduced into the mPC–CE capillary after introduction of the TSB.

During initial studies, we thought that zone band broadening observed in mPC–CE separations of proteins at low pH was due to coexistence of native, denatured and transitional forms of these analytes. To study the extend of these processes on the separation efficiency in mPC–CE, protein standards were dissolved in denaturing matrices such as 8 *M* urea, 8 *M* guanidinium HCl and 20 m*M* dithiothreitol. Proteins in the latter solution were also boiled for 5 min prior to loading onto a C_2 membrane cartridge. The comparison of the electropherograms of protein standards dissolved in water with electropherograms of irreversibly denatured proteins did not show any significant differences in the width of the eluting bands (data not shown). Therefore, contributions from the kinetics of the denaturation process during preconcentration and separation at pH≈3.5 are thought to be minor. Major factors for the quality of the separation remain mPC–CE parameters such as the elution and separation conditions.

From these studies, we can report that mPC–CE is appropriate for the separation of a dilute mixture of proteins. Optimal conditions when using a C_2 impregnated membrane, include use of a polybrene coated capillary, and a BGE of 2 m*M* ammonium acetate in 5% aqueous acetic acid. While protein recovery was low with 60 nl of an elution solvent of 80% acetonitrile in water this volume of solvent was not detrimental to protein separation by mPC–CE. We would also recommend the use of 60 nl of a TSB of 0.5% aqueous ammonium hydroxide, which is followed by a BGE zone of 240 nl to prevent protein readsorption on the membrane. A mPC–CE electropherogram obtained from our standard mixture of proteins using these optimized conditions is shown in Fig. 1.

3.4. Analysis of human aqueous humor by mPC–CE

Aqueous humor (AH) in the posterior and anterior chamber of the eye is a complex mixture of proteins and peptides dissolved in a complex matrix of high concentration of salt [46–48]. The total protein concentration of AH is relatively low (0.5–5.0 mg/ml) and varies from specimen to specimen. The available sample volume is also usually very small

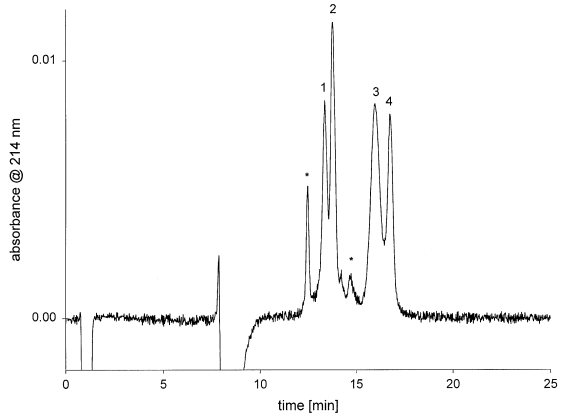

Fig. 1. Separation of a mixture of protein standards (concentration of 0.5 pmol/μl each) by mPC–CE. Injection volume=1 μl. The mPC-cartridge contained a C_2 impregnated Empore membrane. The polybrene coated capillary had dimensions of 57 cm (effective length 50 cm)×50 μm I.D. The BGE was 2 m*M* NH_4OAc in 5% aqueous acetic acid. Proteins were recovered from the C_2 membrane with 60 nl of 80% acetonitrile in water, and 60 nl of a TSB of 0.5% ammonium hydroxide was followed by a zone of 240 nl BGE to prevent protein readsorption on the membrane. The separation potential was −15 kV, and analyte detection was by UV at a wavelength of 214 nm. Protein identification: (1) carbonic anhydrase, (2) myoglobin, (3) human serum albumin, (4) lysozyme, (*) unknown degradation product.

(≤100 μl). Proteins in AH are either synthesized de novo in the ciliary processes, derived from serum by ultrafiltration or a result of catabolic processes within the eye itself [48]. The analysis of the numerous and diverse proteins is of great interest since many are implicated in pathological changes in the eye such as uveitis, cataract or glaucoma. Our interest is focused on the identification of pseudoexfoliation material (PEX) which deposits on the anterior lens capsule. Its formation is accompanied by an aggressive form of glaucoma [49]. CE and mPC–CE methods for analysis of AH, have been developed to aid our attempts to characterize the chemical nature of PEX by identification of its precursors that may be constituents of this fluid or detection of PEX proteins themselves.

CE analysis of AH in polybrene coated capillaries allowed for the efficient separation of a number of components and was described in detail elsewhere [15]. Typically, 9–11 peaks that are representative of the major components of AH were observed in these studies. Therefore, we have evaluated the use of mPC–CE for this application with an expectation that we could also detect minor components of AH along with abundant constituents of this physiologically important fluid. In addition, we expected that the ability to remove salts and other contaminants that may be introduced during surgery would be advantageous to this analysis.

Conditions and parameters found optimal for the separation of dilute solutions of standard proteins by mPC–CE were used directly for the analysis of AH. After loading 1 μl of the specimens the C_2 membrane was washed with BGE to remove inorganic salts and hydrophilic contaminants from proteins and peptides that adhered to this hydrophobic membrane. After elution with 80% acetonitrile in water, the analytes were subjected to focusing and separation by mPC–CE. The resulting electropherograms were characterized by the detection of approximately 15 peaks (Fig. 2). The last peak was always large and we have tentatively assigned it as HSA. The preceding peaks were smaller and sharper and depending on the overall flow-rate and the capillary surface conditions these were either partially or baseline resolved. In many cases, HSA recovery was assessed by the peak area measurements to be ≥90%. We attribute this result to contributions made by the salty

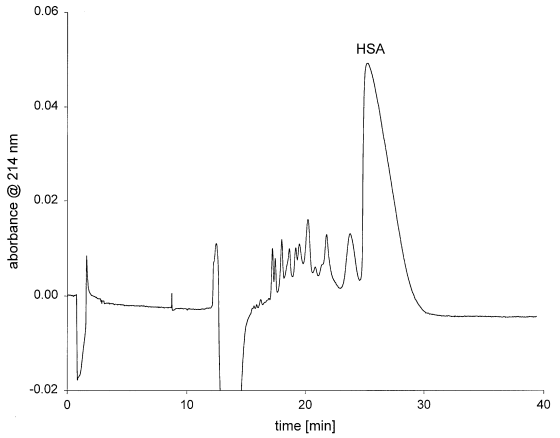

Fig. 2. Separation of human aqueous humor by mPC–CE. Injection volume=1 μl and all other conditions as in Fig. 1 except the capillary length was 77 cm (effective length 70 cm).

sample matrix that may more appropriately condition the C_2 membrane for subsequent elution of the adsorbed proteins. Nevertheless, each specimen was concentrated on a fresh membrane.

Although protein concentrations in some specimens of AH are high enough to allow their separation and detection out of small injected sample volumes (~3 nl) by CE the application of mPC–CE is advantageous for a number of reasons. Direct injection of AH results in separations of high efficiency but limited resolution for CE separations. The efficiency of mPC–CE separations is lower than observed in CE but the overall number of responses as well as the intensity of these peaks is significantly higher. mPC–CE also allows the application of ≥1 μl of sample increasing the probability to observe those proteins which are present at very low concentration. The on-line sample clean-up allows for the removal of hydrophilic sample components and contaminants from surgery implying that almost exclusively proteins and peptides are observed in the electropherograms.

4. Conclusions

Our studies showed membrane based preconcentration is applicable to the analysis of dilute solutions of proteins by CE. The technology requires the use of membranes with hydrophobic characteristics such

as C_2, C_8, C_{18} or SDB. In our studies, the main parameters affecting preconcentration and separation were optimized, using a C_2 impregnated mPC-cartridge, by analysis of a mixture of protein standards. Comparing different elution solvents, 80% acetonitrile yielded best protein recovery. This appears to contradict macroscopic observations where solutions of 80% acetonitrile cause protein precipitation. However, due to the design of the mPC-cartridge the actual concentration of the eluent encountered by proteins on the membrane is most likely lower but sufficient to effect their elution from this device. Observed recoveries were sometimes very low and varied for protein standards ($\leq$25%) and physiological specimens ($\geq$90%). This suggests that the matrix composition of the sample may affect the membrane properties and therefore, analyte adsorption and desorption processes. Postelution focusing of analyte zones is of great importance in mPC–CE. The superimposition of tITP conditions with a pH gradient improves the efficiency and resolution of protein bands. Optimal conditions were achieved using a BGE of 5% acetic acid and 2 m*M* ammonium acetate and applying 60 nl of 0.5% ammonium hydroxide as a trailing stacking buffer after analyte elution.

The optimized mPC–CE method was applied to the analysis of aqueous humor and provided good results for the separation of the components of this physiologically important fluid. Some 15 proteins were enriched and separated in the analyzed specimens. Here, mPC–CE was shown to be an effective method for the on-line removal of matrix components from physiologically derived specimens that enables the detection of proteins which are present at very low concentrations. This methodology reduces sample handling by off-line pretreatments, and thus is likely to have wide applicability in the analysis of physiologically derived fluids and tissue extracts.

Acknowledgements

This work was supported by the AHAF and the Mayo Foundation. The help of Mrs. Diana Ayerhart in the preparation of this manuscript is greatly appreciated. SN also acknowledges Finnigan-MAT for their financial support.

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