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Zone electrophoresis of proteins on a poly(methyl methacrylate) chip with conductivity detection

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Abstract

This work deals with zone electrophoresis (ZE) separations of proteins on a poly(methyl methacrylate) chip with integrated conductivity detection. Experiments were performed in the cationic mode of the separation (pH 2.9) with a hydrodynamically closed separation compartment and suppressed electroosmotic flow. The test proteins reached the detector in less than 10 min under these working conditions and their migration times characterized excellent repeatabilities (0.1–0.6% RSD values). The chip-to-chip agreements of the migration times, evaluated from the ZE runs performed on three chips, were within 1.5%. The conductivity detection provided for protein, loaded on the chip at 10–1000 μg/ml concentrations, detection responses were characterized by 1–5% RSD values of their peak areas. Such migration and detection performances made a frame for reproducible baseline separations of a five-constituent mixture (cytochrome *c*, avidin, conalbumin, human hemoglobin and trypsin inhibitor). On the other hand, a high sample injection channel/separation compartment volume ratio of the chip (500 nl/8500 nl) restricted the resolution of proteins of very close effective mobilities in spite of the fact that in the initial phase of the separation an electric field stacking was applied. A maximum macroconstituent/trace constituent ratio attainable for proteins on the chip was assessed for cytochrome *c* (quantifiable when its concentration in the loaded sample was 10 μg/ml) and apo-transferrin (containing a trace constituent migrating in the position of cytochrome *c* detectable when the load of apo-transferrin was 2000 μg/ml). This assessment indicated that a ratio of 1000:1 is attainable with the aid of conductivity detection on the present chip.
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1. Introduction

Currently, considerable attention is being paid to the development of miniaturized analytical systems (laboratory-on-a-chip) suitable for the separation and/or analysis of proteins (for a review see, e.g., Refs. [1–8]). Here, following the concept of miniaturized total analytical systems (μTAS) [9], electroseparation methods have proved their excellent predisposition for implementation into such analytical systems [5,7,8]. Of the electroseparation methods, zone electrophoresis (ZE) and related techniques [micellar electrokinetic chromatography (MEKC), capillary electrochromatography (CEC), gel electrophoresis (GE)] and isoelectric focusing (IEF) meet best the requirements regarding rapid

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resolution of proteins before their on- or post-column detection [4,5,10–25].

While in the early days of development of the laboratory-on-a-chip systems, the chips were manufactured from glass, quartz and silicon, at present, the use of polymers and plastics has become preferable. In spite of the fact that these materials do not meet perfectly all technical requirements, their use has significant advantages, at least, from the production point of view and attainable price [26,27]. Consequently, besides chips made of glass [12–14,16,17,24,25] and quartz [18,22], also poly(dimethylsiloxane) [10], and poly(methyl methacrylate) [11,19] chips were used for electroseparations of proteinous constituents.

So far, contrary to conventional CE, the use of fluorescence detection techniques dominates monitoring of the separation of proteins performed on CE chips. Although fluorescence offers high detection sensitivities for proteins (usually in combination with suitable labeling reactions), this, in fact, also reflects certain limitations of current chip designs as far as the detection is concerned (see, e.g., recent reviews [7,8,28]). On the other hand, technical solutions providing UV absorbance photometric detection, a preferred detection technique in conventional CE of proteins, suitable for chip-based CE devices appeared recently in the literature [18,19,22]. In one of the quoted works [19], for example, a device integrating UV absorbance detection with a poly(methyl methacrylate) chip is described.

Recently, Galloway et al. [11] showed that an in-channel conductivity detection can be used to monitor the MEKC separation of proteins performed on a poly(methyl methacrylate) chip. Although the use of conductivity detection is not rare in conventional capillary ZE (CZE) [29], this is very likely the first report dealing with its use for the detection of proteins separated by one of the ZE techniques. This, at first glance, surprising fact is understandable when risks linked with disturbances in the response of the conductivity detection in CE [30–32] and the nature of the response of conductivity detection in ZE [33,34] are considered. While the former source of detection disturbances can be eliminated by contactless conductivity sensing [29,35], small fluctuations in the conductivity during the separation due to changes in the composition of the carrier electrolyte solution very likely set limits of the conductivity detection in ZE.

A transfer of some electroseparation techniques into a chip format is the subject of our current research interest [36–45]. Here, using poly(methyl methacrylate) (PMMA) chips of different designs with integrated conductivity detection, we pay attention to ZE [36,42,44], isotachophoresis (ITP) [36,39–41] and ZE with on-line ITP sample pretreatment [36–38,43,45]. One of the PMMA chip designs, intended for ZE and ITP separations, provides an enhanced sample loadability and its use is convenient, e.g., in ZE separations when the electric field driven sample stacking can be effectively employed [42,44]. In this work we investigated the use of this chip for ZE separations of proteins. Reproducible separations of proteins and their quantitation based on the response of the conductivity detection were our main concern. Therefore, to minimize a number of sources that could adversely affect the migration velocities of proteins, we preferred in this feasibility study the use of a hydrodynamically closed separation compartment of the chip and, at the same time, suppressed electroosmotic flow [36].

2. Experimental

2.1. Instrumentation

A poly(methyl methacrylate) chip employed in this work (Fig. 1) was fabricated by a procedure described elsewhere [46]. The separations in this miniaturized device were performed in a laboratory-constructed CE instrument that can be used for both single-column and coupled-column operations [36]. This equipment consisted of two units (Fig. 2): (1) an electrolyte and sample management unit (E&SMU, in Fig. 2), connected via 300 μm I.D. FEP (fluorinated ethylene–propylene copolymer) capillary tubes to the inlets of the channels on the chip. Valves of this unit (V-CE and V-S, in Fig. 2) served to open these inlets on filling the channels and they were closed during the separations. Micropumps (P-CE and P-S, in Fig. 2), connected to the inlets of the corresponding valves, delivered the electrolyte and sample solutions to the channels before the ZE run.

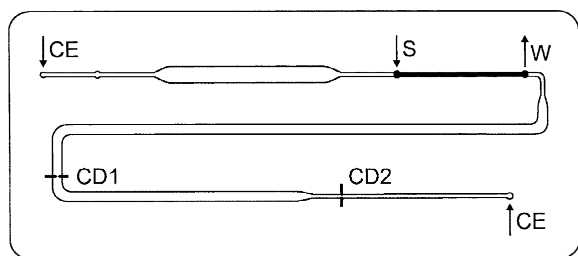

Fig. 1. An arrangement of the channels on the PMMA chip. CE, inlets of the carrier electrolyte solution into the chip channels; S, sample inlet; W, outlet to waste; CD1, CD2, platinum conductivity sensors. A 500-nl sample injection channel is defined by S and W (12.5×0.2×0.2 mm; length×width×depth). The separation channel (W–CD2) consists of two sections: (1) a 5700-nl channel between W and CD1 (59.4×0.5×0.2 mm; the volume is corrected for a transient part behind W) and (2) a 2800-nl channel between CD1 and CD2 (31.4×0.5×0.2 mm; the volume is corrected for a transient part in front of CD2).

An outlet channel of the chip, connected to a waste container (W, in Fig. 2), was permanently opened. (2) An electronic and control unit (E&CU, in Fig. 2) delivered the driving current, and measured con-

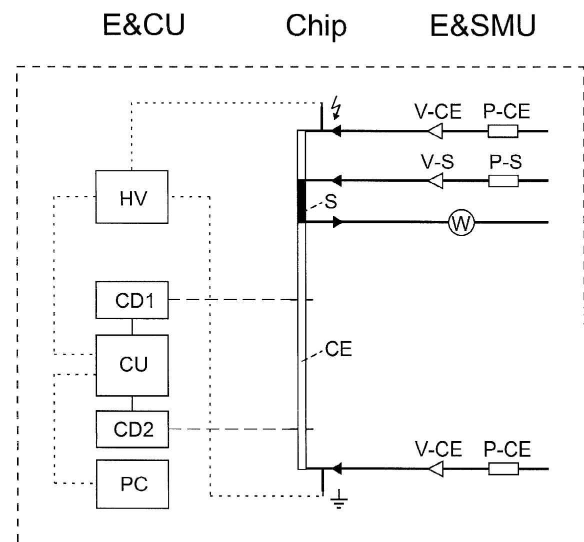

Fig. 2. A scheme of the CE equipment provided with the chip. Electronic and control unit (E&CU): CU, control unit; HV, high-voltage power supply (0–50 μA, 0–5 kV); CD1, CD2, conductivity detectors coupled to the detection sensors on the chip. Electrolyte and sample management unit (E&SMU): V-CE, V-S, valves for the inlets of the carrier electrolyte (CE) and sample (S) solutions into the chip channels, respectively; W, outlet to waste; P-CE, P-S, micro pumps for filling the chip channels with the carrier electrolyte and sample solutions, respectively.

ductivity with the aid of platinum detection sensors, sputtered on the cover of the channels of the chip [46]. It also interfaced the CE equipment to a PC.

ITP Win software (version 2.31), obtained from Kascomp (Bratislava, Slovakia), was used for a time-programmed control of the ZE runs and for the acquisition of the detection data and their processing.

2.2. Electrolyte solutions and samples

Chemicals used for the preparation of the electrolyte solutions and the solutions of model samples were obtained from Sigma–Aldrich (Seelze, Germany), Serva (Heidelberg, Germany), Reanal (Budapest, Hungary), Lachema (Brno, Czech Republic) and Merck (Darmstadt, Germany). A list of the test proteins, along with suppliers of their preparations, is given in Table 1.

Methylhydroxyethylcellulose 30 000 (Serva), purified on a mixed-bed ion-exchanger (Amberlite MB-3, Merck), was used as a suppressor of electroosmotic flow. It was added to the carrier electrolyte solutions or it was applied as a coating of the inner walls of the separation channels. The composition of the carrier electrolyte solution employed in the ZE separations on the chip is given in Table 2.

Water demineralized by a Pro-PS water purification system (Labconco, Kansas City, KS, USA), and kept highly demineralized by a circulation in a Simplicity deionization unit (Millipore, Molsheim, France), was used for the preparation of the electrolyte and sample solutions. The electrolyte solutions were filtered by disposable syringe membrane filters of 0.8-μm pore sizes (Sigma) before use.

The channels of the chip were washed daily with a 0.5% aqueous solution of detergent (Extran MA 03, Merck) containing also sodium azide at a 0.05% (w/v) concentration. They were filled with this solution also when the chip was not in use.

3. Results and discussion

3.1. Transport and separating conditions

For reasons already noted above, we preferred ZE separations of proteins with suppressed electroosmotic (EOF) and hydrodynamic (HDF) flows of the

Table 1
A list of test proteins

Protein	Code	p*I*	M_r	Supplier
Bovine albumin	BSA	4.9	66,000	Sigma–Aldrich
Human albumin	AH	4.9	66,000	Calbiochem, La Jolla, CA, USA
Egg albumin	AE	4.6–4.7	45,000	Serva
apo-Transferrin	AT	5.8	78,000	Sigma–Aldrich
Cytochrome *c*	CC	10.6	12,400	Merck
α-Lactalbumin	LA	4.5	14,200	Sigma–Aldrich
Trypsin inhibitor	TI	4.5	21,000	Sigma–Aldrich
Avidin	A	10.5	66,000	Calbiochem
Conalbumin	C	5.9	76,000	Sigma–Aldrich
Insuline	I	5.3	5730	Sigma–Aldrich
Myoglobin	M	7.4	17,500	Sigma–Aldrich
Lysozyme	L	>11	14,300	Sigma–Aldrich
Trypsinogen	T	9.3	24,000	Sigma–Aldrich
Carbonic anhydrase	CA	5.9	30,000	Calbiochem
Bovine hemoglobin	HB	6.8	64,000	Sigma–Aldrich
Human hemoglobin	HH	6.8	64,000	Sigma–Aldrich
Pig hemoglobin	HP	6.8	64,000	Sigma–Aldrich
Human γ-globulin	G	5.2	–	Imuna, Šarišské Michal'any, Slovak Republic
β-Lactoglobulin B	LB	5.2	35,000	Sigma–Aldrich

carrier electrolyte solution. To reach such transport conditions on the chip, we performed the separations in a hydrodynamically closed separation compartment with a minimized ζ-potential of the walls of the chip channels. Therefore, the inlet channels to the separation compartment of the chip were closed with the aid of valves (V-CE and V-S, in Fig. 2) during the ZE runs (to prevent HDF in the separation compartment). EOF was suppressed by coating the walls by MHEC present in the carrier electrolyte solution in which the separations were performed (Table 2).

A large series of the carrier electrolyte solutions was investigated in a context with the cationic and anionic ZE separations of proteins on the present chip [47]. Some of these carrier electrolytes did not meet our requirements, as the chemicals from which their solutions were prepared had less satisfactory ionic purities (sources of disturbances in the conductivity detection of proteins). The use of some carrier electrolytes was limited due to (very likely) the presence of system zones (migrating discrete disturbances in the carrier electrolyte compositions [48] that were visualized by the conductivity detector). The best results in these respects were achieved with the carrier electrolyte given in Table 2. Here, all the test proteins (Table 1) migrated cationically and they reached the detector (CD2 in Figs. 1 and 2) in less than 10 min. In addition, its composition reflected a sensitive conductivity detection [44,45] of the studied proteins as well (see below).

Table 2
Electrolyte system

Solvent	Water
Carrier electrolyte	Acetic acid
Concentration of the carrier electrolyte (m*M*)	100
Carrier cation	H_3O^+
Counter-ion	Acetate
EOF suppressor	MHEC[a]
Concentration of the EOF suppressor (%, w/v)	0.05
pH	2.9

[a] MHEC, methylhydroxyethylcellulose.

In ZE experiments performed on the present PMMA chips we found that surfaces of the injection and separation channels exhibited detectable adsorptive properties. These were manifested by significant drifts of the detection signals in the ZE runs performed after an operation break (when this lasted about 10 h or more) in the use of the chip (see 1, in Fig. 3). Repeated ZE runs restored the performance of the system (desorbed the adsorbed constituents) with time. A more effective solution in such situations was obtained when immediately after the operation break a ZE run with a proteinous con-

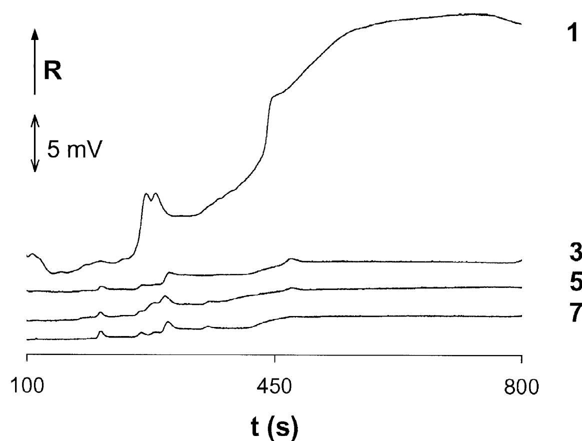

Fig. 3. Conditioning of the separation and sample injection channels on the PMMA chip. Electropherograms were obtained from repeated blank ZE runs with a 10% carrier electrolyte solution loaded into the sample injection channel. 1, A starting blank run (the chip was not in use for about 12 h with the channels filled with water); 3, 5, 7, blank runs following the ZE runs with a proteinous sample (cytochrome *c* (1 mg/ml) dissolved in a 10% carrier electrolyte solution). The ZE runs were carried out in the electrolyte system described in Table 2 with the driving current stabilized at 25 μA.

stituent was performed. Then the required analytical performance of the chip was restored quickly and good repeatabilities (reproducibilities) of blank ZE runs were always reached as illustrated by electropherograms in Fig. 3. Here, very likely, the proteinous constituent acted as a strong displacer of the constituents adsorbed on the channel surfaces.

3.2. ZE separation and quantitation of proteins

Illustrative electropherograms as obtained for some of the test proteins (Table 1) under the preferred transport and separating conditions on the chip (see above) are shown in Fig. 4. From the electropherograms we can see that besides the main constituents the preparations contained also other cationically migrating constituents. The impact of these impurities on the quality of the obtained data were neglected in this work. In this context we should note that the migration velocities of the test proteins (major peaks) exhibited excellent repeatabilities and RSD values of their migration times were in the range of 0.1–0.6% in the separations performed on one chip.

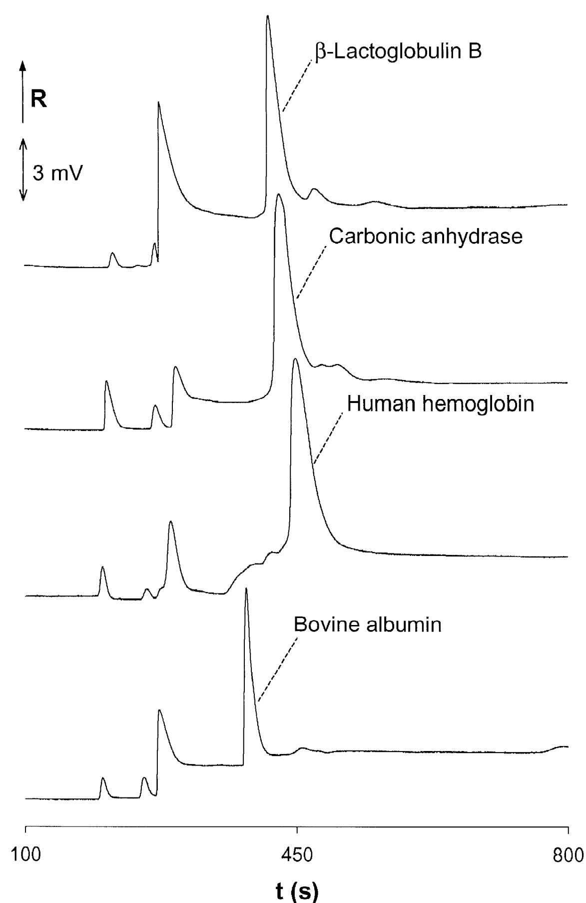

Fig. 4. Electropherograms from the ZE runs of selected proteins on the PMMA chip. The proteins, dissolved at a 1 mg/ml concentration in a 10% carrier electrolyte solution, were loaded on the chip. The ZE runs were carried out in the electrolyte system given in Table 2 with the driving current stabilized at 25 μA.

The test proteins had, under the separating conditions employed, close effective mobilities and, for a particular separation path on the chip (see Figs. 1 and 2), only five of them could be baseline resolved in one ZE run (Fig. 5). Here, a high sample injection channel/separation compartment volume ratio of the chip (500 nl/8500 nl) could significantly contribute to a limited resolution (a high injection dispersion) in spite of the fact that in the initial phase of the separation a ZE sample stacking was effective. Nevertheless, the ZE separations of a test mixture of proteins showed very good repeatabilities as is apparent from electropherograms in Fig. 5 and from

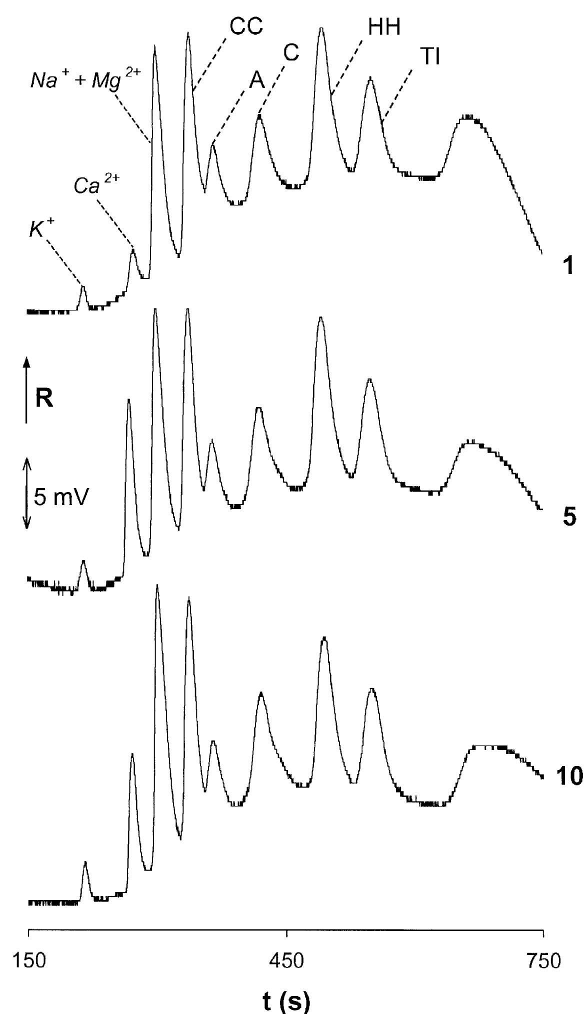

Fig. 5. Electropherograms as obtained from the response of the conductivity detector in repeated ZE separations of a test mixture of proteins on the same PMMA chip. Three electropherograms (1st; 5th; 10th) from a series of 10 repeated ZE separations are shown. The test proteins (cytochrome *c*, CC; avidin, A; conalbumin, C; human hemoglobin, HH; trypsin inhibitor, TI), dissolved (each at a 100 μg/ml concentration) in a 10% carrier electrolyte solution, were loaded into a 500-nl sample injection channel of the chip. The ZE separations were carried out in the electrolyte system described in Table 2 with the driving current stabilized at 25 μA.

the data summarized for the chips employed in this work in Table 3.

Although the conductivity detection cannot be considered as an optimum technique for the detection of proteins in ZE [30–32], it provided in our experiments a very good agreement of the detection response in repeated ZE runs with samples containing individual proteins at 10–1000 μg/ml concentrations (typically 1–5% RSD values for the peak areas). This is also apparent from the data obtained from the separations of proteins on different chips (Table 3). The calibration graphs obtained for four of the test proteins (Table 4) also indicate the capabilities of the present chip in a quantitative work with proteins.

The PMMA chip used in this work can be considered as a disposable device. Therefore, its routine analytical use requires a simple chip-to-chip transfer of the separation and/or analytical procedures. To assess some basic features of the chip in this respect we performed a series of repeated runs on three chips. Electropherograms in Fig. 6 and data summarized in Table 3 show that excellent agreements of the migration data (the mean values of the migration times of proteins on different chips agreed within 1.5%) and electrophoretic profiles were obtained. Such a performance, undoubtedly, reflects precision of the fabrication procedure employed and, to a certain extent, also the transport and separating conditions used in the separations (see above). On the other hand, the detection sensors of the chips exhibited different response characteristics (see the electropherograms in Fig. 6 and the relevant data in Table 3). As no deterioration of performance of the conductivity detection [30,31] due to the presence of proteinous constituents was observed, this can be ascribed to differences in the conductance cell constants of the sensors on various chips (scatters in the measuring geometry in various chip sensors). Here, the use of an appropriately chosen internal standard offers a straightforward solution to this response problem in a chip-to-chip transfer of the analytical procedures.

Model experiments aimed at finding a maximum value for the macroconstituent/trace constituent ratio attainable for proteins on the present chip were restricted by electrophoretic purities of the test proteins (see, e.g., Fig. 4). Nevertheless, our results obtained for cytochrome *c* (quantifiable when its concentration in the loaded sample was 10 μg/ml) and apo-transferrin (containing a trace constituent migrating in the position of cytochrome *c* detectable when the load of apo-transferrin was ca. 2 mg/ml)

Table 3
Repeatabilities of migration and quantitation data of proteins as obtained from the responses of the conductivity detectors in the ZE separations performed on different chips

Protein	Parameter	Chip no. 1		Chip no. 2		Chip no. 3	
		Mean ($n=10$)	RSD (%)	Mean ($n=10$)	RSD (%)	Mean ($n=10$)	RSD (%)
Cytochrome *c*	Migration time (s)	336.3	0.2	339.9	0.3	340.5	0.3
	Peak area (mV s)	229.3	3.2	131.6	3.7	183.1	11.9
	Peak height (mV)	15.4	4.2	8.8	6.0	12.1	7.4
Avidin	Migration time (s)	363.5	0.3	367.2	0.3	368.2	0.5
	Peak area (mV s)	97.6	3.9	64.3	8.1	77.4	15.3
	Peak height (mV)	5.5	3.4	3.8	5.6	4.5	19.9
Conalbumin	Migration time (s)	419.9	0.1	416.4	0.5	422.1	0.3
	Peak area (mV s)	182.6	4.7	95.5	13.8	113.6	2.5
	Peak height (mV)	6.9	4.8	3.6	8.5	4.2	4.8
Human hemoglobin	Migration time (s)	493.9	0.2	495.4	0.4	499.7	0.4
	Peak area (mV s)	246.1	1.0	152.7	3.6	181.6	1.3
	Peak height (mV)	10.9	1.5	6.3	4.8	7.8	1.9
Trypsin inhibitor	Migration time (s)	547.9	0.2	555.1	0.3	556.6	0.6
	Peak area (mV s)	173.3	4.5	119.1	4.6	133.0	4.1
	Peak height (mV)	7.0	3.0	4.8	5.4	5.4	5.1

n, Number of repeated runs with the test sample; the test sample contained each protein at a 100 μg/ml concentration.

revealed that a ratio of 1000:1 should be attained with the aid of conductivity detection on the present chip. An electropherogram in Fig. 7 illustrates these possibilities of the present chip in trace protein analysis. Here, however, it is also apparent that a combination of the chip with a more sensitive detection technique can become, especially, beneficial for this category of (bio)analytical problems.

4. Conclusions

The results of this feasibility study show that the present PMMA chip, providing higher sample loads, offers working conditions suitable to reproducible separation of proteinous constituents. In the light of the results of our previous work with a similar chip design [36] it seems logical to assume that the attained migration performance was, at least in part, due to the use of a hydrodynamically closed separation compartment and suppressed electroosmotic flow.

The contact conductivity detection provided a reliable and sufficiently sensitive technique in monitoring the ZE runs with test proteins in this work. Our results obtained with this detection technique indicate that with appropriately designed detection sensors (e.g. microstrips sputtered in the chip channels [46]) and perfectly de-coupled measuring electronics [31], its performance can be free of disturbances due to the separated (proteinous) constituents. However, this cannot be considered as general, as interactions of some proteins with the electrode surfaces cannot be excluded [32] and disturbances

Table 4
Regression equations of the calibration graphs ($y = a + bx$) for the quantitation of proteins from the response of the conductivity detector

Protein	Regression equation	r	Concentration span (mg/ml)	n
Cytochrome *c*	$y = 464.2 + 3011.4x$	0.9985	0.1–1	7
Conalbumin	$y = -14.5 + 1375.9x$	0.9971	0.1–1	7
Human hemoglobin	$y = 26.7 + 1368.4x$	0.9918	0.03–0.3	7
Trypsin inhibitor	$y = 0.5 + 1167.4x$	0.9983	0.1–1	7

n, Number of data points; *r*, correlation coefficient; *y*, peak area (mV s); *x*, concentration (mg/ml).

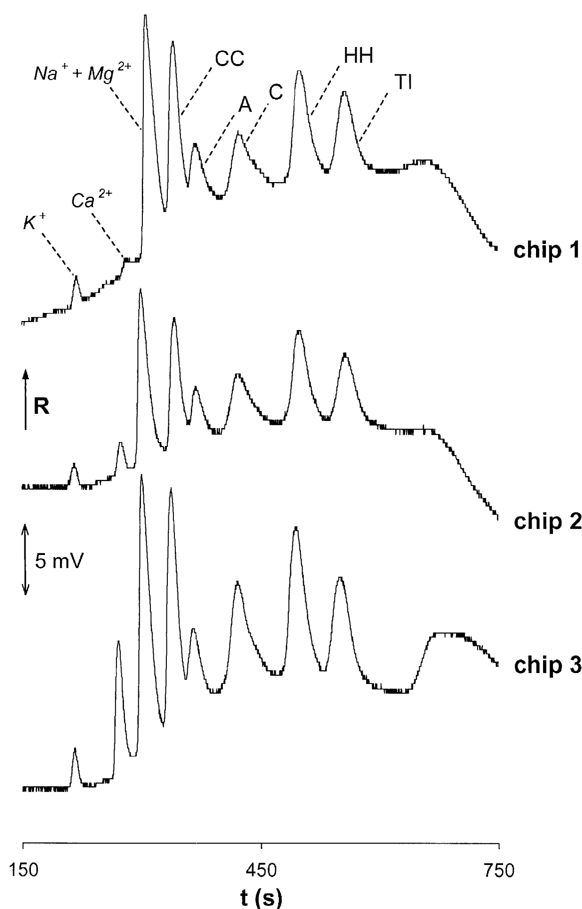

Fig. 6. A chip-to-chip reproducibility of the ZE separations of proteins. The test proteins (cytochrome *c*, CC; avidin, A; conalbumin, C; human hemoglobin, HH; trypsin inhibitor, TI), dissolved (each at a 100 μg/ml concentration) in a 10% carrier electrolyte solution, were loaded into 500-nl sample injection channels of the chips. The ZE separations were carried out in the electrolyte system described in Table 2 with the driving current stabilized at 25 μA. Additional information can be found in Table 3.

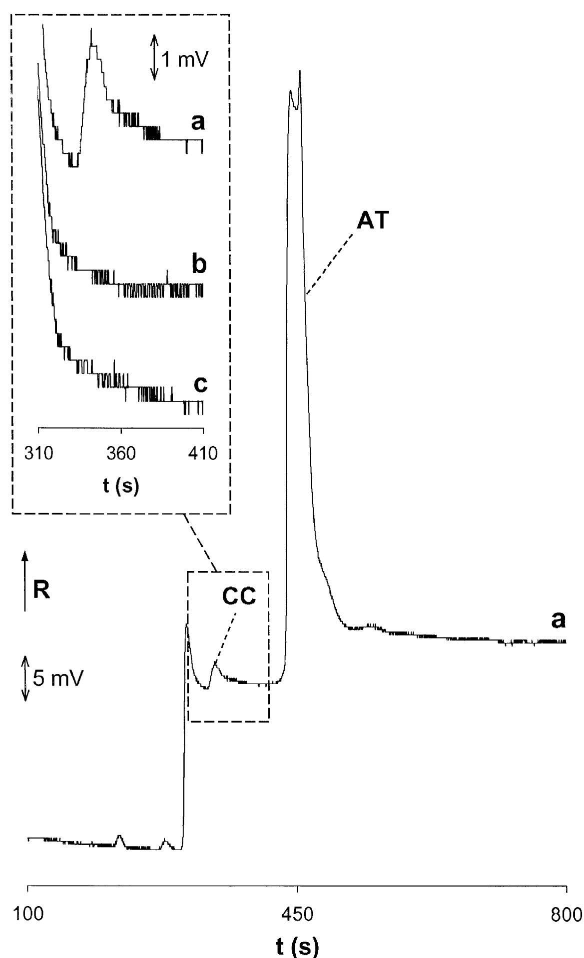

Fig. 7. A ZE separation of proteins present in the loaded sample at significantly differing concentrations. (a) An electropherogram from the separation of the sample containing cytochrome *c* (10 μg/ml) and apo-transferrin (1000 μg/ml), dissolved in a 10% carrier electrolyte solution; (b) a segment of an electropherogram from the migration position of cytochrome *c* when the loaded sample contained only apo-transferrin (1000 μg/ml); (c) a segment of an electropherogram from the migration position of cytochrome *c* as obtained from a blank ZE run (a 10% carrier electrolyte solution was loaded). The ZE separations were carried out in the electrolyte system described in Table 2 with the driving current stabilized at 25 μA.

attributable to these interactions [30,31] can occur. In this context it seems appropriate to mention that other concepts of contact conductivity sensing [49] or the use of contactless conductivity detection [29,35] can eliminate these problems in a general sense.

A low selectivity of the conductivity detection in ZE and an inherent response of this detection technique to system peaks [48] make it a priori less suitable in situations when the proteins present in a multicomponent mixture are to be detected. On the other hand, in such instances it can serve as a useful technique in identifying system disturbances, as these need not be detected by selective detectors [48]. Therefore, its use on the chip can be very beneficial in a combination with a suitable selective

detector (e.g. photometry light absorbance, fluorescence) in the way favored in some conventional CE equipment [31,50].

Our results indicate that the present PMMA chip, providing sub-μl sample loads, may be useful in determining purities of proteins and determining trace proteins present in samples containing a large excess of a limited number of proteins. Here, the macroconstituent/trace constituent ratio of 1000:1 seems attainable when the conductivity detection is employed. However, a significant enhancement of this ratio can be expected by transferring the present procedure into the ITP–ZE combination performed on the column-coupling chip of closed design [36–38,43,45].

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