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Selective protein removal and desalting using microchip CE☆

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Abstract

This paper describes the on-line sample pretreatment and analysis of proteins and peptides with a poly(methylmethacrylate) (PMMA) microfluidic device (IonChip™). This chip consists of two hyphenated electrophoresis channels with integrated conductivity detectors. The first channel can be used for sample preconcentration and sample clean-up, while in the second channel the selected compounds are separated. Isotachophoresis (ITP) combined with zone electrophoresis (CZE) was used to preconcentrate a myoglobin sample by a factor of about 65 before injection into the second dimension and to desalt a mixture of six proteins with 100 mM NaCl. However, ITP–CZE could not be used for the removal of two proteins from a protein/peptide sample since the protein zone in the ITP step was too small to remove certain compounds. Therefore, we used CZE–CZE for the removal of proteins from a protein/peptide mixture, thereby injecting only the peptides into the second CZE separation channel.

Keywords: 2D microfluidic device; Isotachophoresis-zone electrophoresis; Sample depletion; On chip sample pretreatment; Proteins and peptides

1. Introduction

In a time where mankind has revealed the human genome and is finding ways to unravel the human proteome, the challenges, generated in biology, are becoming less easy to solve with standard analytical methods. Faster and more effective separations with preferably integrated sample clean-up procedures are demanded. Combining different areas of expertise, separation sciences and microfluidics technology, provide us with tools to solve the problems mentioned above, at least partly. Although liquid chromatography (LC) has also been configured to chip dimensions just recently, e.g. [1,2], the focus seems to be on the more easy implementation of electrophoretic separation methods on a microfluidic device. On-chip analysis offers certain advantages over conventional systems: decrease in analysis time, parallel analysis, possibility of automation, the use of smaller sample volumes, reduced reagent/buffer use, lower production costs, portability, and the main advantage seems to be the possibility to integrate several functions on the chip, thereby bringing the lab to the sample [3,4]. Although these systems work perfectly well for the analysis of small organic and inorganic compounds, the challenge is the analysis of complex biological samples, especially the targeting of proteins in samples like blood, urine or plasma. In the last 10–15 years, several papers have been published on the analysis of whole proteins in different microchip materials, mainly glass [5–8], quartz [7] and polymers including polydimethylsiloxane (PDMS) [4,9,10] and poly(methylmethacrylate) (PMMA) [3,11–14].

When using microchips for analysis, detection is one of the major problems since the channels usually have a low internal diameter. Considering the small sample amounts often used as well, UV detection is not the method of first choice. Therefore, more sensitive detection methods are used like fluorescence [3,4,7,8,15–17], mass spectrometry (MS) [6,14] and electrochemical detection methods [10,11–13,18–20].

Another crucial step in the analysis of biological samples when using CE, is the sample pretreatment including, e.g. removal of salt and other compounds as well as sample preconcentration. Several sample preparation steps have been implemented on chip, e.g. microdialysis [21,22], free-flow elec-

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trophoresis [23,24], sample stacking [25–27], isotachophoresis [18–20], solid-phase extraction [16,28] and liquid–liquid extraction [15,29].

The PMMA microchip device described in this paper, the IonChip™, was originally designed for fast and sensitive analysis of low amounts of small molecules to be analyzed in the ITP [30,31], the CZE [30], the ITP–ITP [31], the ITP–CZE [18–20,30,32] or the CZE–CZE mode [33]. One of the unique features of this IonChip™ is the possibility to couple two CE modes in a single analysis. Although several 2D separation systems on chip have been published, combining e.g. sample stacking and CZE [27,34,35], ITP and CZE [12,18–20,30–32,36,37], isoelectric focusing (IEF) and CZE [38], open-channel electrochromatography (OCEC) and CZE [17], solid-phase extraction (SPE) and OCEC [39], micellar electrokinetic chromatography (MEKC) and CZE [40], SPE–MEKC [41], or even in-line SPE–CZE–MS [42], only few describe the analysis of intact proteins [12,34,37,38] or protein digests [27,40].

Ölvecká et al. analyzed proteins with the IonChip™, but found that removal of some major proteins from a mixture of proteins was difficult in the ITP–CZE mode [12]. For removal of proteins from a protein/peptide mixture we will therefore focus on CZE–CZE as an alternative for ITP–CZE. Prior to on-line CE separation, different sample pretreatment steps can be carried out on this device, e.g. preconcentration, removal of salts and other compounds. Furthermore, the IonChip™ has a relatively large sample loop (900 nL) and channel diameter (140–200 μm by 200–500 μm) which, in combination with the stacking in ITP and the possibility of removing matrix compounds before injection into the second dimension, increases the sample loadability of the system. This could be of benefit for, e.g. the search for low concentrations of smaller compounds (drugs/peptides) or proteins in biological matrices or trace constituents in food products [18,32]. Moreover, it is a low cost, accessible and user-friendly device.

The present paper focuses on the use of the IonChip™ with conductivity detection for sample preparation and subsequent separation, where the ITP–CZE mode is used for preconcentration of myoglobin and for the removal of salt from a protein mixture. Moreover, the CZE–CZE mode is tested for the removal of proteins from a protein/peptide mixture.

2. Materials and methods

2.1. Chemicals

Acetonitrile (gradient grade) was obtained from Riedel-de-Haën (Seelze, Germany). Formic acid was purchased from Merck (Darmstadt, Germany). The electrolyte solutions were prepared by the Department of Analytical Chemistry of the Comenius University (Bratislava, Slovak Republic) and were all extra pure. The carrier electrolyte consisted of 20 mM acetic acid without and with 0.1% hydroxyethylcellulose (HEC), with a pH of 3.20. Leading electrolyte: 20 mM ammonium acetate, 2.18 mM acetic acid, 0.05% HEC, pH 5.73. Terminating electrolyte: 10 mM acetic acid, 0.1% HEC, pH 3.36. Angiotensin I, angiotensin II, angiotensin III, neurotensin, bradykinin, human serum albumin (HSA, p*I* = 4.7; Mw = 66,478 Da), lactoferrin (Lac, from bovine milk, Mw = approx. 90 kDa), β-lactoglobulin B (Lac B, from bovine milk, p*I* = 5.3, Mw = 18,276 Da), myoglobin (Myo, from horse skeletal muscle, p*I* = 7.3 (major component) and 6.8 (minor component), Mw = 17.6 kDa), avidin

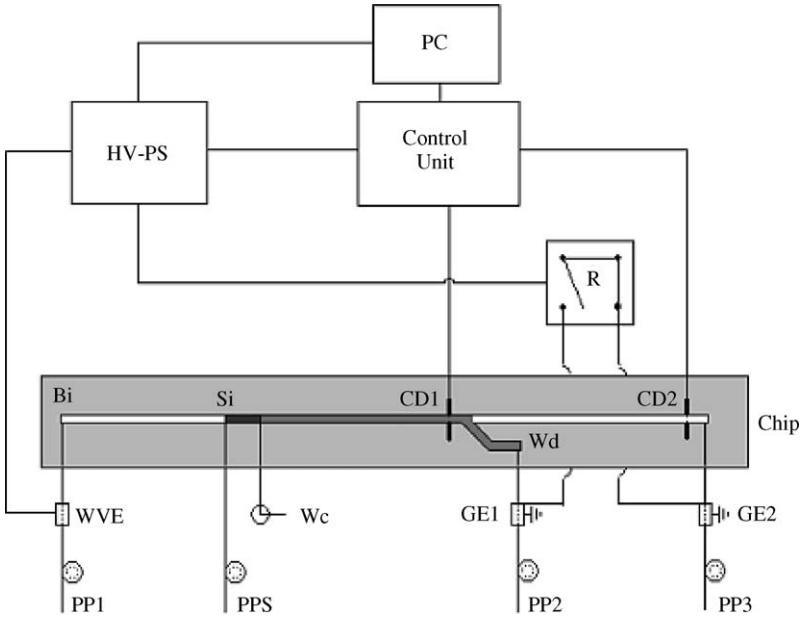

Fig. 1. Schematic overview of Ionchip™ column coupling device. A high-voltage power supply (HV-PS; 0–50 μA; 0–7 kV) controls the high-voltage electrode (HVE) located near peristaltic pump 1 (PPl). The chip is filled with electrolyte solution through peristaltic pumps 1–3 (PPl, PP2 and PP3), sample is injected from PPS. Excess liquid is directed to a waste channel (We). The current from the HV-PS is switched by a relay (R) to either the first ground electrode (GEl) directing compounds to the waste on the chip (Wc), or to the second ground electrode for separation (GE2). Conductivity detectors monitor the sample before (CD1) and after removing compounds (CD2). The whole IonChip™ system is controlled by the control unit and can be operated by a computer (PC).

(Avi, from egg white, pI = 10, Mw = 66 kDa) and cytochrome *c* (CC, from horse heart, pI = 10.0–10.5; Mw = 12,384 Da) were obtained from Sigma-Aldrich (Steinheim, Germany). All solutions were prepared with water taken from a Milli Q water purification system (Millipore, Bedford, MA, USA).

2.2. ITP–CZE and CZE–CZE

ITP–CZE and CZE–CZE experiments were carried out with the IonChip^{TM}, a PMMA microchip device, previously described by Kaniansky et al. [36] and manufactured by Merck KGaA (Darmstadt, Germany). This device consists of (1) the electronic and control unit and (2) the electrolyte and sample handling unit (Fig. 1). The latter unit is equipped with three membrane driving electrodes: one high voltage electrode (HVE, 0–7 kV, 1–50 μA) and two ground electrodes (GE1 and GE2). Furthermore, four peristaltic pumps (PP1, PP2, PP3 and PPS) are included in the system and are used for filling and flushing the chip. Before an ITP–CZE run, the chip is filled with electrolyte solutions and sample solution through the peristaltic pumps: pump 1 (terminating electrolyte), pump 2 (leading electrolyte), pump 3 (carrier electrolyte) and pump S (sample). When performing a CZE–CZE run, pumps 1–3 fill the chip with carrier electrolyte. While flushing the system, excess liquid is directed to a waste channel (Wc). During the electrophoretic runs, the current is kept constant (30–20 μA in the ITP–CZE runs and 20 μA in the CZE–CZE runs). The current is controlled by the polarity of the potential applied between the high voltage electrode and ground electrode 1 (for the first ITP or CZE mode, before and during removal of compounds) or ground electrode 2 (after removal of compounds, separation step). The switching is controlled by a relay (R). Removal of compounds is accomplished by switching the direction of the driving current. First the current is applied between the high voltage electrode and ground electrode 1. When the first conductivity detector (CD1) reaches a predefined threshold value (200 mV in the ITP–CZE runs or 15 mV in the CZE–CZE runs), a preprogrammed time lag (the column switching time or CST) starts counting down, while the driving current is maintained between the high voltage electrode and the first ground electrode, thus directing all the compounds that pass the T-split or bifurcation into the waste channel. After this column switching time, the direction of the current is changed and applied between the high voltage electrode and ground electrode 2, thereby injecting all the compounds that have not passed the bifurcation into the second separation channel. The electronics also controls the peristaltic pumps and interfaces of the device and connects these to a computer. Filling and running of the chip as well as data acquisition and processing is enabled through the use of MicroITP software version 1.0, developed by the Department of Analytical Chemistry of the Comenius University (Bratislava, Slovak Republic). With this software it is also possible to acquire time programmed control of the ITP–CZE and CZE–CZE runs, based on the signal of CD1.

The identities of the compounds in the electropherograms were determined by running each compound separately. Next mixtures were analyzed by adding a single compound to the previous sample, thus eventually injecting all peptides and proteins in a single sample.

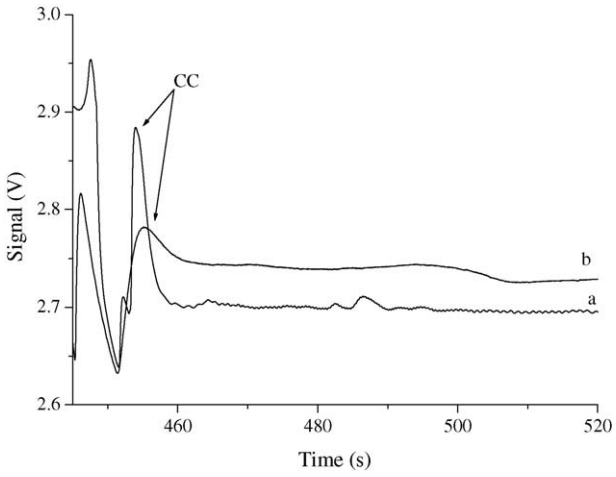

Fig. 2. ITP–CZE electropherogram (recorded at CD2) of a sample containing cytochrome *c* (CC, 100 μg/mL) with (trace a) and without (trace b) HEC in the buffer. Conditions as described in Section 2.2.

3. Results and discussion

3.1. Preconcentration and desalting using ITP–CZE

When using the ITP–CZE mode, the IonChip^{TM} is filled with leading, terminating and carrier electrolyte as described in Section 2.2. The conductivity of the background electrolyte influences the migration times and has an effect on the response of the detectors. In the ITP–CZE as well as in the CZE–CZE experiments we used hydroxyethylcellulose (HEC) in the background electrolytes, since it suppresses the electroosmotic flow (EOF), it reduces adsorption to the wall and minimizes dispersion [31], especially for certain proteins that are known to adsorb to capillary walls like cytochrome *c*. Fig. 2 shows electrophero-

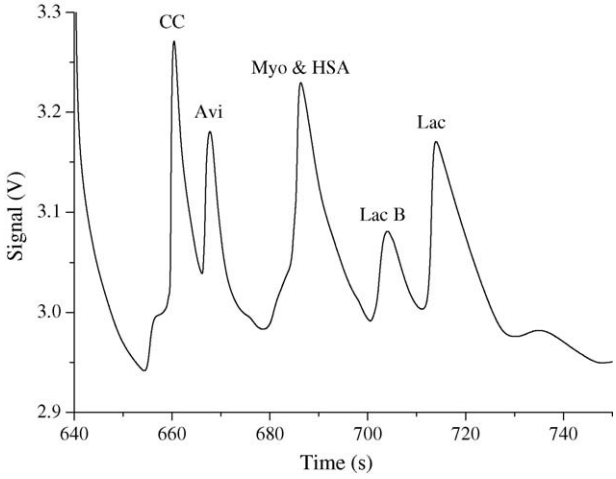

Fig. 3. ITP–CZE electropherogram (recorded at CD2) of a sample containing cytochrome *c* (CC, 100 μg/mL), avidin (Avi, 333 μg/mL), myoglobin (Myo, 100 μg/mL) HSA (100 μg/mL), β-lactoglobulin B (Lac B, 100 μg/mL) and lactoferrin (Lac, 100 μg/mL). Conditions as described in Section 2.2.

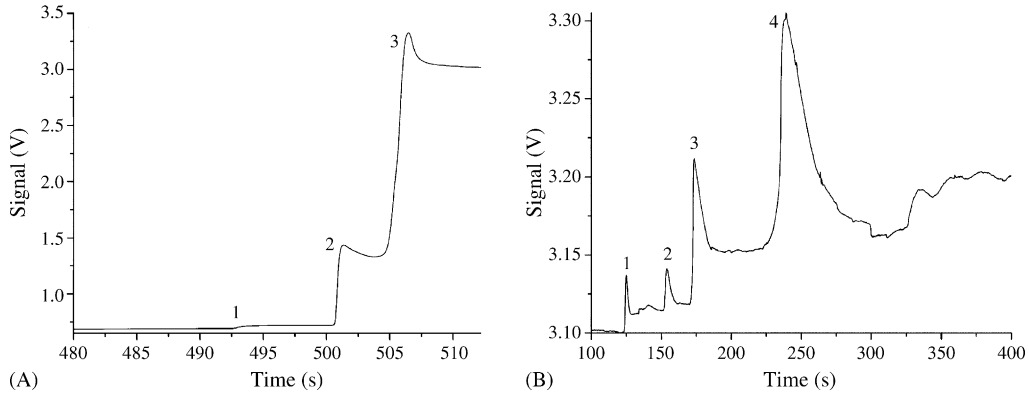

Fig. 4. ITP–CZE (A) and CZE–CZE (B) electropherograms recorded at CDl during the ITP step (A) and the first CZE (B) step for a sample containing 125 μg/mL myoglobin. The third increase in signal in (A) and peak 4 in (B) represents myoglobin passing the detector. Conditions as described in Section 2.2.

grams of ITP–CZE runs of cytochrome *c* without and with using HEC in the background electrolyte. The peak corresponding to cytochrome *c* is much broader when using a buffer without HEC.

ITP–CZE of a mixture of proteins was performed to show that proteins can be analyzed with this system, in a similar way Ölvecká et al. [12] did before. The possibility of monitoring the ITP step with a conductivity detector in the first channel of this system makes it relatively easy to reproducibly switch fractions from the first to the second channel, because of the staircaselike signal and the sharp zones created in ITP. Fig. 3 depicts the separation of the protein mixture by ITP–CZE recorded at the second detector. Most of the proteins are separated and show relatively small peaks, although two of the proteins, myoglobin and HSA, were not separated, probably due to the short channel length in which separation after the ITP step takes place.

When performing ITP–CZE on a myoglobin sample of 125 μg/mL, we were able to preconcentrate this protein by a factor of approximately 65 before it was injected into the CZE separation channel. Fig. 4A depicts an electropherogram recorded at CD1 during the ITP step. The plug containing myoglobin (3) passes CD1 in 4 s, resulting in a peakvolume of 13.2 nL. Compared to the injection volume of 900 nL, a concentration factor of 65 was obtained before injecting the myoglobin into CZE separation channel. When running the same sample in the CZE–CZE mode, thereby replacing the leading and terminating electrolyte with carrier electrolyte, our peakvolume at CD1 (Fig. 4B) was 277 nL before injection into the second separation channel. Compared to the first step in a CZE–CZE system, the plug of myoglobin in the ITP step of ITP–CZE is approximately 20 times smaller, thereby increasing the loadability and the concentration sensitivity of the CZE system when using ITP as the first step, indicating that on-chip preconcentration is also a strong feature of this device. These findings were in line with the findings of Ölvecká et al. [12], where an improvement of the concentration limit of detection of 20–50 was found for different proteins when using ITP–CZE instead of CZE.

A main problem in the electrophoretic analysis of biological samples is the presence of high concentrations of salt. When the IonChip^{TM} is operated in the ITP–CZE mode, it also serves as a fast on-line desalting device with subsequent electrophoretic separation. Since small ions have a higher mobility than large charged molecules, salts can easily be removed from a mixture of proteins by directing the salts to the waste channel. Fig. 5 shows a typical ITP–CZE electropherogram recorded at CD2 of a sample containing proteins in the presence of 100 mM NaCl. A large salt plug is present in front of the small protein plug when the salt is not removed (Fig. 5a, using a CST = 1s). Furthermore, the proteins are not separated and migrate as one plug. When the salt is removed (Fig. 5b) during the ITP step (CST = 16s, directing the salt to Wc), the proteins can be separated in the second channel.

3.2. Protein removal using CZE–CZE

When using ITP–CZE for a more complex sample, however, it turns out to be problematic to remove two proteins completely from the sample containing five peptides and two proteins. Ölvecká et al. [12] already came to the same conclusions using a sample of six proteins and attributed it to the fact that the protein stack, after the ITP part, is very small. Since one

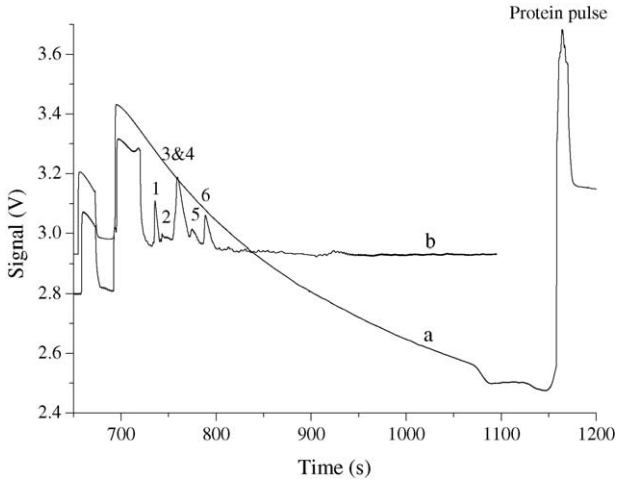

Fig. 5. ITP–CZE electropherogram (recorded at CD2) of a sample containing cytochrome *c* (1; 100 μg/mL), avidin (2; 333 μg/mL), myoglobin (3; 100 μg/mL) HSA (4; 100 μg/mL), β-lactoglobulin B (5; 100 μg/mL) and lactoferrin (6; 100 μg/mL) in the presence of 100 mM NaCl with a CST of 1 s (not desalted, trace a) and 16 s (desalted, trace b). Conditions as described in Section 2.2.

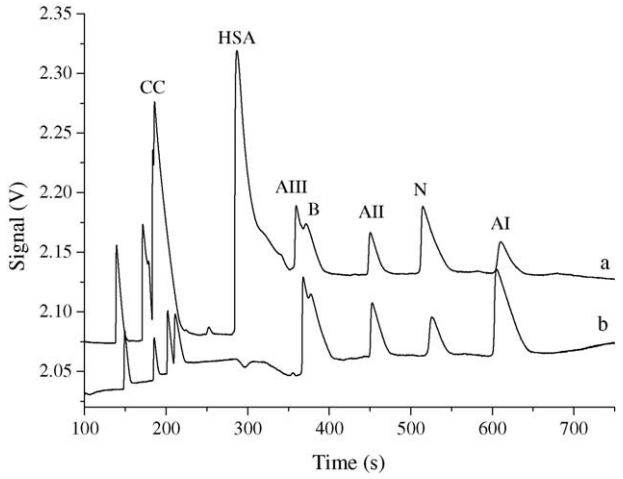

Fig. 6. Electropherogram of a sample containing five peptides (10 μg/mL of each of the following peptides: angiotensin I (AI), angiotensin II (AII), angiotensin III (AIII), bradykinin (B) and neurotensin (N)) and two proteins (100 μg/mL CC and 300 μg/mL HSA) recorded at CD2 in the CZE–CZE mode with a CST of 1 s (no removal of proteins, trace a) and 120 s (removal of proteins, trace b). Conditions as described in Section 2.2.

of our goals was to remove certain proteins without affecting the rest of the sample, we have chosen for CZE–CZE. In this mode, however, we lacked the advantage of sample-preconcentration of ITP. Fig. 6 shows electropherograms recorded at CD2, with (CST = 120 s) and without (CST = 1 s) the removal of the two proteins from the sample. The two proteins are removed from the sample, while the five peptides are injected into the second CZE separation channel and recorded at CD2. Therefore, this method could be useful for sample clean-up and reduction of the complexity of a biological sample, e.g. removing high abundant proteins like albumin, before a separation step. Although not specifically designed for protein analysis, the strong points of this chip, preconcentration and sample clean-up prior to an electrophoretic separation, can also be used for protein and peptide samples.

4. Conclusions

Although not designed for protein analysis it is demonstrated that the IonChip™, is a very powerful device for protein analysis, combining sample pretreatment and separation in a single run. Due to the relatively large sample loop (900 nL) and channel diameter, combined with the isotachophoretic preconcentration, the loadability of the system is relatively high compared to conventional microchip CE. In the ITP step of the ITP–CZE mode, a concentration factor of approximately 65 was obtained for myoglobin prior to injection into the second separation channel. Compared to CZE–CZE, the volume of the plug injected into the second CZE step was approximately 20 times smaller when using ITP–CZE. In the ITP–CZE mode we were also able to remove a high salt concentration present in a protein mixture, thereby increasing the amount/concentration of components to be injected onto the second separation channel. The novelty presented in this paper is the use of CZE–CZE instead of ITP–CZE for the removal of proteins from a protein/peptide mixture, since ITP–CZE could not remove the proteins from this mixture. Although compromising the enrichment factor in the CZE–CZE mode, removal of two proteins from a protein/peptide mixture was accomplished. This again demonstrates the sample preparation possibilities of this device.

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