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Microfluidic electrocapture interfaced with electrospray mass spectrometry

Susanne Vollmer, Juan Astorga-Wells, Tomas Bergman, Hans Jörnvall *

Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77 Stockholm, Sweden

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Abstract

Microfluidic electrocapture of peptides and proteins in an inert capillary with electric contacts via conductive membranes is useful for sample handling before mass spectrometry. The use of electrocapture has already been demonstrated for sample clean-up, pre-concentration, chemical modification and peptide separation, all without the need for supporting gels or chemical binding. This method allows multiple micro-reactions, extensive peptide separations and work with membrane proteins from detergent-solubilized samples. Until now, electrocapture has utilized MALDI mass spectrometry, but here we demonstrate that it can be interfaced with electrospray ionization and hence with on-line mass spectrometric analysis of peptides separated from protein digests. These applications combined with the present on-line approach show electrocapture to be a versatile technology with great potential.

Keywords: Microfluidic electrocapture; Electrospray ionization mass spectrometry; Interface; Peptide separation

1. Introduction

Microscale flows have unique properties and can be used in components and instrumentation beneficial to biological sciences [1]. Microfluidic devices provide advantages in sample handling, reagent mixing, separation and detection [2–9]. In proteome analysis, the sample complexity and the low abundance of many proteins provide methodological challenges. In this context, microfluidic electrocapture technology offers novel approaches. It captures molecules in a flow-stream by counterbalancing hydrodynamic and electric forces without the use of supporting gels or chemical binding, hence reducing handling losses.

Initially, electrocapture was found to be useful in sample clean-up, buffer change and pre-concentration, both before capillary electrophoresis [10] and MALDI mass spectrometry [11]. We then found that once captured, proteins can be submitted not only to direct subsequent elution (by switching off the voltage) but also to chemical reactions, such as reductions, alkylations and proteolytic fragmentation, by proper additions to the flow-stream before the elution. In this manner, on-line micro-reactions can be used to produce desired chemical modifications before analysis [12]. Similarly, by addition of coenzymes and substrates to the flow-stream, enzymatic reactions can be monitored [13]. Presumably, other bindings, such as those of hormones, xenobiotics, or further biologically interesting molecules should be possible to monitor in the same manner.

Next, we found that elution of peptides and proteins does not need to be performed as a single step, where all proteins are eluted at once, but can be carried out gradually by the use of a decreasing voltage gradient. Peptides are then separated and eluted mainly according to increasing electrophoretic mobility [14] but the separation is influenced also by other parameters. Thus, electrocapture should in principle be useful both for pre-analytical sample preparation and for peptide separation before mass spectrometry. With the applications already demonstrated, one could even visualize a work-flow where electrocapture applied to a membrane protein preparation [14] is used for, in turn, removal of the detergent [11], cysteine alkylation [12], fragment generation and separation of peptides generated [14], all by successive steps in one device before final mass spectrometric analysis. For routine use in such an ideal flow scheme, further steps are required and are being developed, suggesting future progress in electrocapture use.

* Corresponding author. Tel.: +46 8 52487702; fax: +46 8 337462.
E-mail address: Hans.Jornvall@ki.se (H. Jörnvall).

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One limitation has been combination of the electrocapture technique with electrospray ionization in the mass spectrometric analysis. Such an interface has been difficult to design for both spatial and electric reasons, but would be necessary for on-line mass spectrometric analysis of peptides separated in the electrocapture step much like it is for the combination of capillary electrophoresis with mass spectrometry [8,15]. We have managed to combine separation methods of the electrocapture cell with an electrospray source, allowing direct analysis of separated peptides in a quadrupole time-of-flight tandem mass spectrometer, rather than manual collection of discrete fractions to MALDI targets as before. The use of model proteins demonstrates that on-line analysis can be achieved. The present interface design may be possible to improve and several parameters, such as the effect of temperature need further studies. However, the results demonstrate the feasibility of the transfer step and of a combination of systems that provide direct analysis of peptide samples separated in the electrocapture cell.

2. Materials and methods

2.1. Reagents and chemicals

Myoglobin, hemoglobin, bovine serum albumin (BSA), cytochrome C, dithiothreitol (DTT), iodoacetamide (IAA) and ammonium formate were obtained from Sigma, acetonitrile from Rathburn Chemicals, Scotland. Porcine sequencing grade modified trypsin was from Promega and the water used was from a MilliQ water purification system.

2.2. Proteins and pre-treatments

Stock solutions for the proteins were prepared at concentrations of 5 mg/ml for myoglobin, bovine serum albumin and cytochrome C, and 1 mg/ml for hemoglobin. Carbamidomethylation of cysteine residues was carried out by mixing 25 μl of protein with 5 μl of 45 mM DTT and subsequent incubation at 50 °C for 15 min. After cooling to room temperature, IAA (5 μl of 100 mM) was added and the mixture incubated for 15 min at room temperature. Following alkylation, reagents were diluted by addition of ammonium bicarbonate (64 μl of 7.5 mM, pH 8), and for digestion 1 μl of trypsin (1 μg/μl in 50 mM acetic acid) was added. The mixture was left at 37 °C overnight and the resulting tryptic digest then stored at −20 °C. Before use, the digest was diluted with 5 mM ammonium formate (pH 5.5), 30% acetonitrile.

2.3. Electrocapture cell

The microfluidic device was manufactured as described in previous publications [10,11]. On a piece of PEEK tubing (127 μm i.d. and 512 μm o.d. Upchurch, Oak Harbor, WA) two small openings 2 cm apart were made with a scalpel and were covered with a conductive tubular cation-selective membrane of poly (tetrafluoroethylene-sulfonate) material (Permapure Inc., Toms River, NY) having an inner diameter of 330 μm and an outer diameter of 610 μm. The covered openings constitute the electric junctions and each was placed into a separate electrode chamber made from 500 μl Eppendorf tubes (Hamburg, Germany).

2.4. Electrocapture system

The electrocapture cell, made in-house as given above, was placed into an electrocapture instrument (Biomotif AB, Danderyd, Sweden). The main components are a syringe pump with a 250 μl syringe, a microinjector, a voltage-gradient power supply and a holder keeping the electocapture cell and electrodes in place. The syringe pump and power supply are controlled via an in-house developed software which monitors voltage and current during each run and which is also used to program different voltage gradients. The instrument was operated with the anode located at the upstream electric junction of the cell. Electrodes of platinum-wire were placed into the electrode chambers which were filled with 100 mM ammonium formate (pH 5.5). The pump syringe was filled with 5 mM ammonium formate (pH 5.5), 30% acetonitrile and provided a continuous hydrodynamic flow. The sample was injected into the system via a 1.25 μl loop of the microinjector, introducing 1.1 pmol hemoglobin and for the tryptic digest mixture, 2.5 pmol myoglobin, 27.5 pmol cytochrome C and 3.75 pmol bovine serum albumin. The device, injector and interface are shown in Fig. 1. Pre-manufactured cells have the same measures, but electricity and all tubings are then shielded within Perspex (Fig. 1B), with electrodes locked at correct positions through a lid construction, all for laboratory-use electric safety.

2.5. Mass spectrometry

Mass spectra were acquired using a Micromass/Waters Q-TOF 1 instrument (Micromass, Manchester, UK) operated in the positive ion mode. The electrocapture cell was connected to the mass spectrometer via a fused silica capillary (50 μm i.d., 27 cm length) between the outlet of the cell and the tip emitter (NewObjective, SilicaTip FS360-20-10-D-20-C7). The fused silica capillary was inserted into the microfluidic channel and the other end into the tip emitter. The capillary voltage was kept between 1.1 and 2.2 kV.

2.6. System operation

The general operation in establishing the interface involved three steps: injection of protein digest, capture of peptides injected, and voltage reduction to sequentially release peptide fractions for ESI-MS analysis. In order to keep interferences from the mass spectrometer as low as possible, a stable spray was established at 1.1 kV before elevating the voltage in the electrocapture device. The sample was injected and pumped into the electrocapture device at a flow rate of 0.2 μl/min and a voltage of 150–170 V. To ensure that the entire sample was injected and captured the initial voltage was maintained for 30–40 min before starting the stepwise voltage reduction gradient. Reduction steps were programmed using the in-house developed software. Separated peptide fractions emerging from the cell were transported

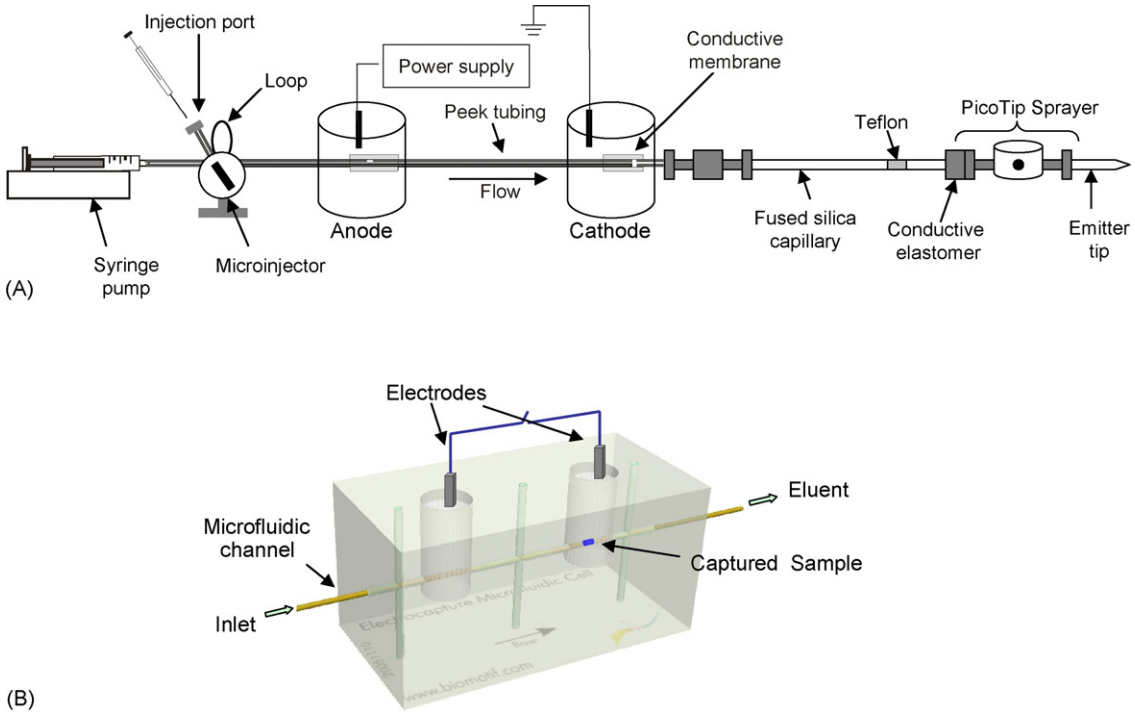

Fig. 1. Electrocapture cell, injector and interface set-up for peptide capture and separation, connected on-line to electrospray mass spectrometry (A). Recently available pre-manufactured cell (B).

directly into the emitter tip of the Q-TOF instrument via the fused silica capillary. Sample and buffer were degassed for 35 min before injection, the sample by placement in an evacuated desiccator and the buffer by sonication. Proper safety precautions were taken since both pieces of equipment operate at very high voltages. The maximum output of the power supply was in the μA range.

2.7. Warning

With the conditions specified, we achieved peptide separation in the electrocapture cell that was maintained in the interface and produced interpretable mass spectra. However, a warning is necessary. Should these test conditions be repeated elsewhere, the electricity hazard in an open configuration constitutes a serious danger and requires maximal caution.

3. Results

3.1. Establishment of the interface set-up

Initially, blank runs were performed using the new set-up of the capture cell on-line (Fig. 1A) with the electrospray source to validate the system. In attempts to find a buffer or solution suitable for both the electrocapture and the electrospray processes, we found that 5–10 mM ammonium formate (pH 5.5), 30% acetonitrile was sufficiently volatile to establish a spray employing a fused silica tip with distal coating in the electrospray unit. This buffer also provides a stable and sufficiently high current profile to allow capture and separation of peptides in the electrocapture cell. The length of the fused silica capillary tubing chosen to connect the two units was initially set at 1 m to avoid electric interference between the two units. For the same reason, we also found that the ionic strength of the ammonium formate should be kept low, and we selected 5 mM.

Next, we tested to capture and separate peptides for analysis by direct transfer into the mass spectrometer via the capillary reaching into the electrospray source. Initially, we did not get good peptide separation but elution as a single set of peaks. We concluded that the then long capillary might cause diffusion of the sample during transfer, reversing the process of fractionation. We therefore shortened the capillary in a stepwise manner to find the optimal length that minimized diffusion but still kept the system free of electric interference. The final length meeting these requirements was 27 cm at an inner diameter of 50 μm.

3.2. On-line sample analysis

During these experiments, we used two protein preparations, one, a mixture of three proteins as given in Section 2, and one, human hemoglobin only. Both preparations were traced by definition of their fragment patterns after reduction, alkylation and tryptic digestion in the usual manner. These pre-conditions can be handled in the electrocapture cell, as shown before [12] but to allow present emphasis on the on-line interface testing, we used pretreated protein solutions, already alkylated and digested as given in Section 2. The on-line experiments therefore started with injection of these digests into the electrocapture cell and capture in the usual manner. The peptides were then gradually released in separation mode (Fig. 2) and analyzed on-line by ESI mass spectrometry.

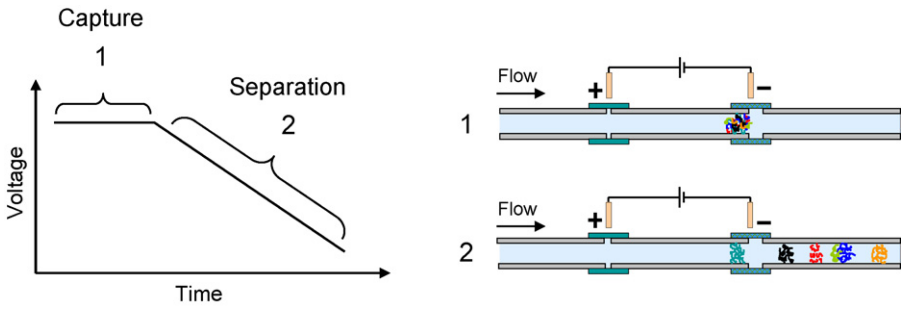

Fig. 2. Scheme of the electrocapture-based separation and sample preparation.

The hemoglobin digest was captured at 75 V/cm. As seen in Fig. 3A–D, different peptide profiles appear in each fraction analyzed after voltage reduction showing successful direct transfer of separated peptides into the mass spectrometer. Similarly, the digest of the mixture of three proteins was captured at 85 V/cm, and also produced the expected separation and subsequent on-line analysis pattern (Fig. 4A–D). Panel A corresponds to the first visible peak in the total ion chromatogram (TIC) 2 min after starting the voltage reduction. Panels B, C and D correspond to the highest TIC peaks, at 2.5, 3.5 and 12.5 min after the start of the voltage reduction. For each peak assignment, *m/z* value, charge state, monoisotopic mass and amino acid sequence

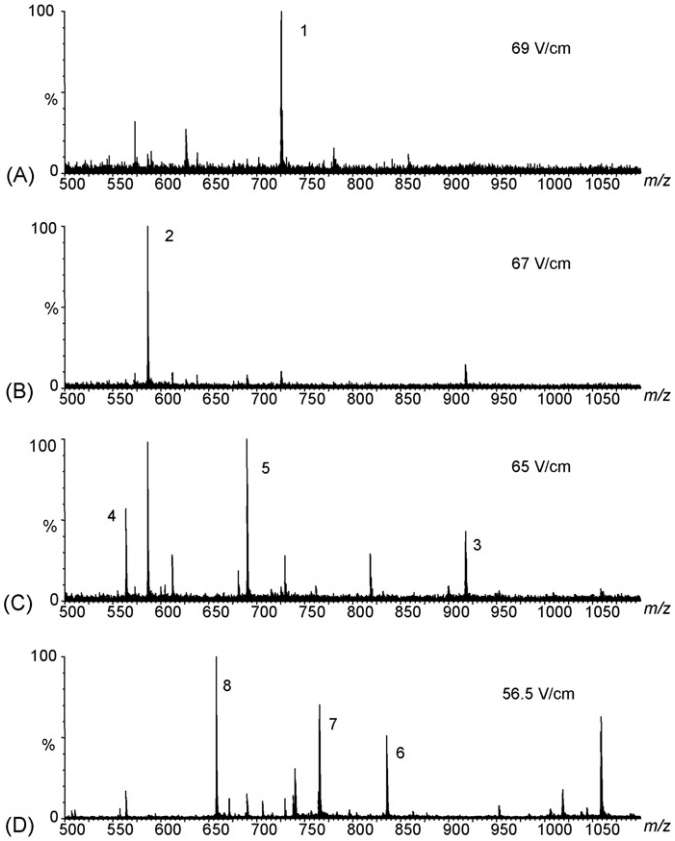

Fig. 3. Electrospray mass spectra of tryptic peptides of human hemoglobin (0.88 pmol/μl) after capture and separation in 5 mM ammonium formate (pH 5.5), 30% acetonitrile. The sample was injected at 0.2 μl/min and the peptides were captured at an initial electric field strength of 75 V/cm for 40 min. Well separated peptides emerging at 69, 67, 65 and 56.5 V/cm are shown (A–D).

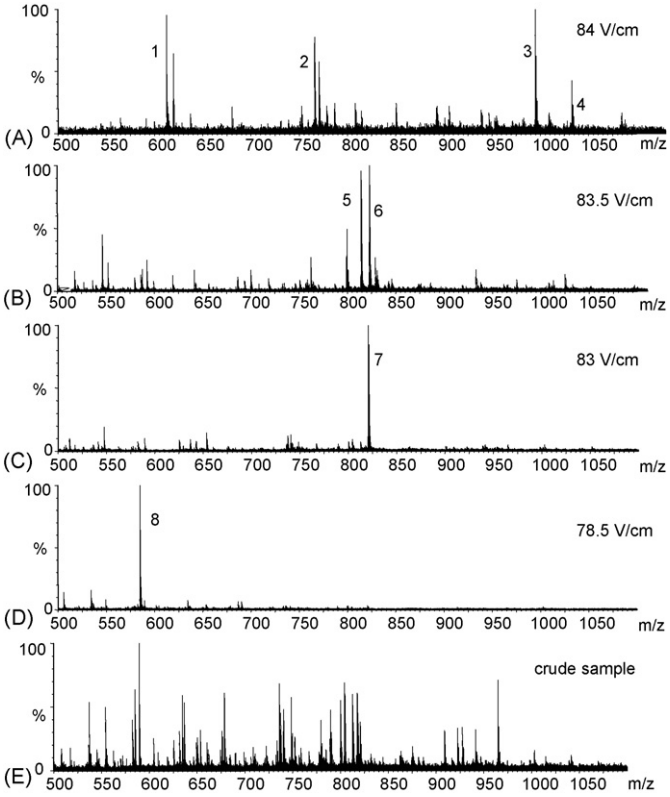

Fig. 4. Electrospray mass spectra of a tryptic digest of a protein mixture containing myoglobin (2 pmol/μl), bovine serum albumin (3 pmol/μl) and cytochrome C (22 pmol/μl) after capture and separation in 5 mM ammonium formate (pH 5.5), 30% acetonitrile. The sample was injected at 0.2 μl/min and the peptides were captured at an initial electric field strength of 85 V/cm for 30 min. Well separated peptide fractions emerging at 81.5, 80.5, 80 and 78.5 V/cm during the voltage gradient are shown (A–D). Crude sample injected at 0.2 μl/min without the capture and separation steps (E).

are given in Tables 1 and 2. The peptides appear to be released mainly according to their electrophoretic mobilities (falling p*I* values) but other parameters may also influence the release.

4. Discussion

In this report, we demonstrate that it is possible to construct a workable interface between microfluidic electrocapture technology and an on-line connected electrospray source for mass spectrometric analysis of peptide separations obtained. The two units were successfully interfaced via a fused silica capillary

Table 1
Molecular properties of peptides corresponding to the numbered peaks in Fig. 3 from a tryptic digest of hemoglobin analyzed by on-line electrospray mass spectrometry after electrocapture and separation

Electric field (V/cm)	Assigned number	*m/z*	Charge state	Monoisotopic mass	Sequence
69.0	1	725.41	+2	1449.79	VVAGVANALAHKYH
67.0	2	586.35	+2	1171.66	VLSPADKTNVK
65.0	3	917.49	+2	1833.89	TYFPHFDLSHGSAQVK
	4	563.80	+2	1126.56	LHVDPENFR
	5	689.88	+2	1378.70	EFTPPVQAAYQK
56.5	6	835.48	+2	1669.89	VLGAFSDGLAHLDNLK
	7	765.41	+2	1529.73	VGAHAGEYGAEALER
	8	657.88	+2	1314.66	VNVDEVGGEALGR

Table 2
Molecular properties of peptides corresponding to the numbered peaks in Fig. 4 from a tryptic digest of a protein mixture containing myoglobin (yielding peptide 5), bovine serum albumin (yielding peptides 1, 4 and 7) and cytochrome C (yielding peptides 2, 3, 6 and 8) analyzed by on-line electrospray mass spectrometry after electrocapture and separation

Electric field (V/cm)	Assigned number	*m/z*	Charge state	Monoisotopic mass	Sequence
84.0	1	582.41	+2	1163.48	LVNELTEFAK
	2	736.00	+2	1469.00	TGQAPGFTYTDANK
	3	964.73	+1	964.73	EDLIAYLK
	4	1002.7	+1	1002.77	LVVSTQTALA
83.5	5	804.05	+2	1607.10	VEADIAGHGQEVLIR
	6	812.53	+2	1624.06	DDPHACYSTVFDK
83.0	7	820.62	+2	1640.24	EETLMEYLENPKK
78.5	8	584.90	+2	1168.80	TGPNLHGLFGR

of 50 μm inner diameter and 27 cm length under the voltage conditions specified. A suitable buffer system was established in the form of 5 mM ammonium formate (pH 5.5), 30% acetonitrile, and different peak profiles were obtained for different voltages. Digests of several proteins and protein mixtures were analyzed, and in all cases, both the peptide separation step in the electrocapture cell and the ESI analysis were successful. We are aware that these results are limited in spread regarding proteins analyzed (we have tested 10-odd proteins), but establishment of the interface conditions was our main emphasis. A comparison to separations by capillary electrophoresis is not relevant until further optimization has been carried out in pre-manufactured and electrically shielded, safe electrocapture cells. However, the two methodologies differ, and in capillary electrophoresis there is no on-line immobilization event. With the results from the present experiments and with regard to successes reported in combinations of capillary electrophoresis and mass spectrometry [8,15,16], we feel confident that an efficient on-line method for peptide fractionation by electrocapture separations followed by peptide identification by tandem mass spectrometry can be obtained. Together with previous results on peptide separations and MALDI mass spectrometry of membrane protein preparations [14], the present data show that multiple and novel approaches to peptide analysis are possible with the electrocapture technology. Independently of future possibilities, electrocapture is currently used in ordinary sample clean-up and concentration before MALDI mass spectrometry, capillary electrophoresis and other analytical micro-techniques.

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