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Capillary electrophoresis–matrix-assisted laser-desorption ionization mass spectrometry of proteins

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Abstract

An "off-line" combination of capillary electrophoresis (CE) with matrix-assisted laser-desorption mass spectrometry (MALDI-MS) has been developed for the structural characterization of CE-separated peptides and proteins. Using a sheath flow interface, similar to that developed for "on-line" CE–fast atom bombardment MS and CE–electrospray MS, an efficient sample isolation procedure has been developed which is applicable to bioorganic compounds in aqueous buffer solutions. This isolation procedure, with subsequent transfer to the MALDI-MS sample target, has been successfully used for the direct analysis of CE-separated proteins of M_r up to 67 000, and a mixture of apolipoprotein AII monomer and homodimer, using sample amounts of less than 1 pmol.

1. Introduction

Capillary electrophoresis (CE) [1] —based on "free zone electrophoresis", which was described and automated by Hjertén in the late sixties [2]— has been developed in recent years as a powerful, high-resolution microanalytical separation method for the characterization of biological macromolecules such as proteins and nucleic acids [3]. The detection and characterization of separated compounds is usually performed by photooptical detectors (UV, diode array, fluorescence detectors). More recently, mass spectrometry (MS) has been combined with CE, mainly "on-line", using direct interfaces with electrospray ionization (ESI) and fast atom bombardment (FAB) MS [4,5]. Sample isolation of CE-separated components has been performed by different methods. A buffer-saturated membrane [6] has been used, followed by derivatization for fluorescence spectroscopy or with immunological detection. Another method utilized a fraction collector, which automatically changes the outlet buffer vessel during the CE run [7]. A similar manual procedure was used for combining CE with ^{252}Cf plasma desorption (PD) MS for the MS characterization of peptides and small proteins, including a combination with Edman degradation and MS peptide mapping [8]. A "coaxial sheath-flow" interface, similar to those used for on-line CE–ESI-MS [4] and CE–FAB-MS [5], has been used for the "off-line" determination of hydrophobic peptides by CE–PD-MS using organic solvents in the CE buffer

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[9] and for the combination of CE and matrix-assisted laser-desorption (MALDI) MS [10,11].

In this study, the application of "coaxial sheath-flow" isolation was used for the combined CE–MALDI-MS analysis of peptides and proteins. Memory effects during the fraction collection step were minimized by using high sheath-flow-rates. Due to the large collection volumes, the transfer of samples onto the MALDI-MS sample-target was carried out "off-line", after lyophilization and addition of matrix. The use of aminopropylsilylated capillaries [12] and a low-ionic-strength acetic acid buffer reduced peak broadening in MALDI-MS by avoiding alkali ion adduct formation. The advantages and problems of an internal calibration with proteins for the combination of CE and MALDI-MS have been investigated, and applications of this "off-line" interface to mixture analysis of apolipoprotein AII are demonstrated.

2. Experimental

2.1. MALDI-MS

A VG Analytical/Fisons TofSpec was used for the acquisition of all mass spectra. It is equipped with a 337 nm nitrogen laser and an accelerating voltage of 24 kV was used. A saturated "matrix solution" of α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI, USA) (recrystallized from methanol prior to use) was made in a solution of 2-propanol–water–formic acid (3:2:1) as described by DeLlano *et al.* [13]. The peptide samples were prepared for MALDI by mixing 1 μl peptide solution in 0.1% trifluoroacetic acid (TFA) with 9 μl "matrix solution", and applying 2 μl onto the sample target. The ApoAII samples were prepared by mixing 0.5 μl of matrix solution with 0.5 μl (1 to 10 pmol/μl) protein solution in 0.1% TFA directly on the target. All samples isolated from CE were vacuum dried and redissolved in 1 μl "matrix solution", which was directly applied onto the target. Crystallization on the target was observed under a microscope and accelerated by gently blowing cold air from a hair dryer over the target surface. For spiking the sample with internal standard, a solution of standard protein (1 pmol/μl myoglobin in 2-propanol–water–formic acid, 3:2:1, v/v/v) was added to the crystallized sample matrix mixture on the target, mixed and crystallized again.

2.2. Capillary electrophoresis

Fused-silica capillaries (Polymicro Technology, Phoenix, AZ, USA) were derivatized with aminopropyltrimethoxysilane (APS) (Aldrich) as described by Moseley *et al.* [12]. Total capillary length was between 1.1 and 1.25 m. The APS capillaries proved to be stable, when stored at pH 3.4 (10 m*M* acetic acid), for several months. When a reduction in the electroosmotic flow was noted, the capillary was flushed with 10 m*M* HCl for 15 min and then equilibrated with 10 m*M* acetic acid. This treatment recovers most of the electroosmotic flow by removing the adsorbed protein and protonating the aminopropyl groups at the capillary surface.

The CE instrument was built in the laboratory using a reversible-polarity, high-voltage power supply (Spellman High Voltage Electronics, Plainview, NJ, USA). A small area of the polyimide coating was burned off with a glowing wire to form a window 15 cm from the end of the capillary for UV detection at 200 nm with an SSI-500 UV detector (State College, PA, USA). The outlet end of the capillary was placed in a stainless-steel tee, and fed through a 20 mm × 700 μm O.D. × 400 μm I.D. steel needle until it protruded approximately 0.3 mm from the end. The steel needle was grounded and served as the ground for the CE system. Prior to use, the APS column was flushed with 10 m*M* HCl for 15 min and with 10 m*M* acetic acid for 30 min. Dilute acetic acid (10 m*M*, pH 3.4) was used as the CE buffer and the sheath flow solvent. After loading the sample onto the capillary by hydrostatic injection (see Table 1), a negative 30 kV voltage and a sheath flow of 20 to 25 μl/min from a pressure bomb [14] were applied simultaneously. The elution time of the UV-detected peak was calculated from the migration time to the detector multiplied by the ratio of the total capillary

Table 1
Sample loading conditions

Peptides/proteins	Concentration (in water)	Loading time (20 cm height) (s)	Amount of sample loaded
Peptide mixture (Fig. 2)	150 pmol/μl each peptide	10	*ca.* 1 pmol
Lysozyme (Fig. 3)	70 pmol/μl (*ca.* 1 μg/μl)	20	*ca.* 1 pmol
Cytochrome *c* (Fig. 4)	80 pmol/μl (1 μg/μl)	20	*ca.* 1.1 pmol
Bovine serum albumin (Fig. 5)	14 pmol/μl (1 μg/μl)	20	*ca.* 200 fmol
Apo AII (Fig. 6)	220 pmol/μl (*ca.* 2 μg/μl)	10	*ca.* 1.5 pmol

The loaded sample amount was calculated from flow-rate measurements at a certain applied hydrostatic pressure on a 1.1 m × 75 μm I.D. capillary (10 s at 20 cm = 7 nl volume loaded).

length:capillary length to the detector. Droplets containing the separated fractions were collected in a vial, vacuum dried, and redissolved in 1 μl of "matrix solution" for MALDI target preparation (see above).

2.3. Peptides and proteins

All peptides [human angiotensin I, luteinizing hormone-releasing hormone (LHRH), bradykinin, Arg^8-vasopressin, oxytocin, sleep-inducing peptide] were purchased from Sigma (St. Louis, MO, USA) as free bases or acetate salts. Proteins [lysozyme from chicken egg white, horse skeletal muscle myoglobin, horse heart cytochrome *c*, bovine serum albumin (fatty acid free) and human apolipoprotein AII (ApoAII)] were from Sigma. All solvents were of analytical grade, water was obtained from a Milli Ro/Milli-Q system (Millipore, Bedford, MA, USA).

2.4. Dithiothreitol reduction

ApoAII was reduced by dissolving it in a 100 m*M* dithiothreitol (DTT)–25 m*M* ammonium hydrogencarbonate solution (in water, pH 8.5) and incubating for 2 h at 37°C. The reaction was stopped by lyophilization. Most of the DTT was evaporated by redissolving the sample in 25 m*M* hydrogencarbonate buffer and relyophilization.

2.5. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Phast-System (Pharmacia, Uppsala, Sweden). Stacking gel; 13 mm, 7.5% T/2% C[a]: separating gel zone; 20% T/2% C, containing 30% ethylene glycol. The SDS-buffer strips contained 0.2 *M* tricine, 0.2 *M* Tris, and 0.55% SDS at pH 8.1. ApoAII was treated with SDS-sample buffer: 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.0, 2.5% SDS and heated to 35°C for 5 min. Staining was performed with Coomassie Blue. Non-reduced and DTT-reduced (1% DTT in sample buffer) ApoAII were compared. The non-reduced sample showed one major band at M_r 17 000 and a minor band at $M_r \approx 8000$. The reduced sample only showed one band at $M_r \approx$ 8000.

3. Results

To evaluate an optimum sheath-flow-rate for sample isolation using the coaxial sheath-flow interface, a mixture of six peptides was separated by underivatized CE. Prior to the sheath-flow experiments, however, the sensitivity of MALDI-MS for the model peptides was evaluated. The sensitivity results are summarized in Table

Table 2
Sensitivity of MALDI-MS for peptides with different acidities

Peptide	Amino acid sequence	Molecular mass	Net charge[a] at pH 3.4	Total amount of peptide on MALDI target (fmol)	S/N ratio in MALDI-MS spectrum
Angiotensin I	DRVYIHPFHL	1297	+2.8	80	20
Bradykinin	RPPGFSPFR	1061	+2.1	80	5
LHRH	pE[b]-HWSYGLRPG-NH_2	1182	+2.0	80	25
Arg^8-Vasopressin	CYIQNCPRG-NH_2	1084	+2.1	80	10
Oxytocin	CYIQNCPLG-NH_2	1007	+1.1	80	2
Sleep-inducing peptide	WAGGDASGQ	849	−0.3	8000	5[c]

[a] Net charge at pH 3.4; sum of net charges of the single amino acids. The net charge is proportional to the proton affinity of the peptide.
[b] pE = Pyroglutamic acid.
[c] Ion abundances of $(M + Na)^+$, $(M + K)^+$ and $(M + H)^+$ are similar.

2, and show that the basic peptides (angiotensin I, bradykinin, LHRH, Arg^8-vasopressin) can be detected with considerably higher sensitivity than acidic peptides (oxytocin, sleep-inducing peptide). For preparing samples for MALDI-MS analysis directly from the effluent of the CE capillary, low sheath-flow-rates are required (2–4 μl/min). Using a 75 μm I.D. × 360 μm O.D. CE capillary and a 400 μm I.D. × 700 μm O.D. stainless-steel needle at the end of the interface (see Fig. 1), such flow-rates proved to be too low to form a droplet during the peak elution time of 10–30 s, which made it impossible to easily collect one droplet per eluting peak. Low sheath-flow-rates also resulted in memory effects at the end of the metal capillary, even if the peaks are baseline separated. To avoid these memory effects at the tip of the stainless-steel needle, sheath-flow-rates of 20 to 25 μl/min were used. This necessitated concentration of the eluted sample solution prior to target preparation for MALDI-MS.

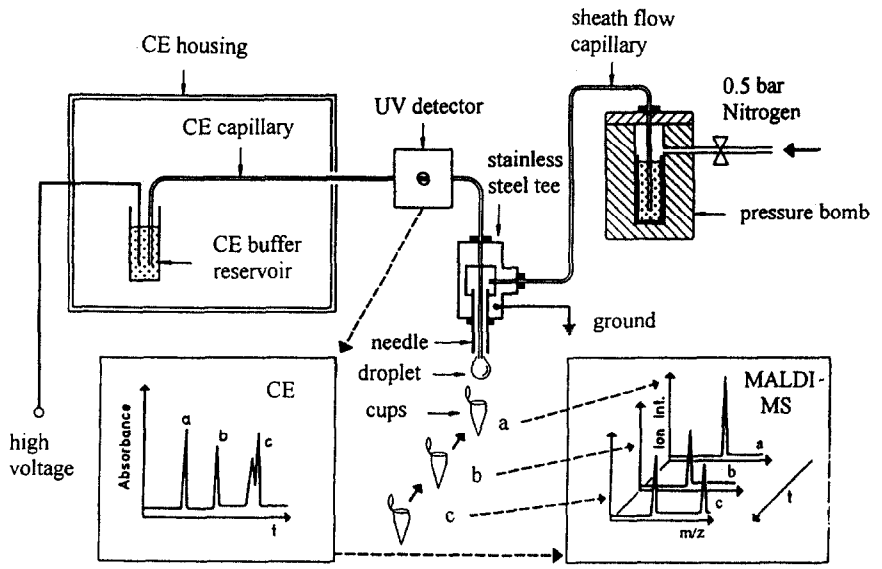

Fig. 1. Scheme of the CE–MALDI-MS interface using a sheath flow.

An equimolar mixture of six peptides (angiotensin I, LHRH, bradykinin, Arg^8-vasopressin, oxytocin, sleep-inducing peptide, Table 2) was partially separated by CE (Fig. 2a). Fractions were collected using a sheath-flow-rate of approximately 20 μl/min, and the collected fractions were lyophilized, redissolved in the matrix solution and the mass spectra were acquired. Only five peptides, four of which were not baseline separated, could be detected by MALDI-MS. Oxytocin gave only a weak signal, and sleep-inducing peptide was not detected at all by MALDI-MS. This was due to either the low sensitivity under the MALDI conditions for acidic peptides (Table 2) or to interference of the background signals with the signal from the peptide. LHRH was detected in fraction F1, and also after elution of the main fraction F1 in fraction F2. This can be explained by the high sensitivity of MALDI-MS for basic peptides.

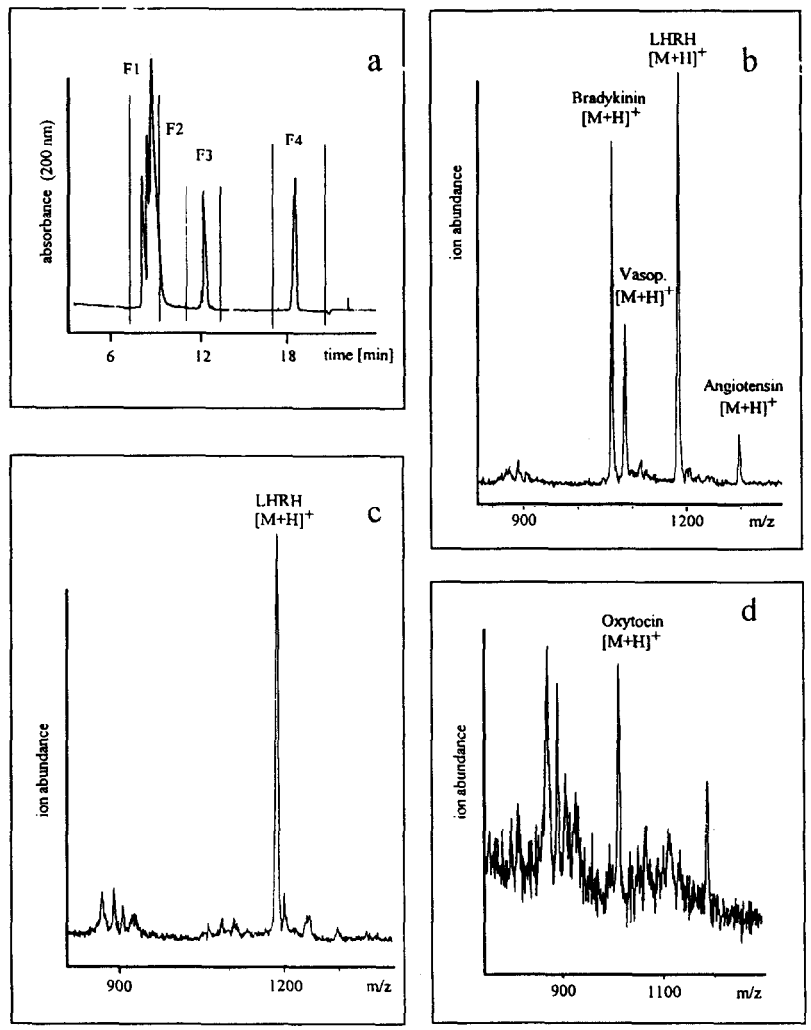

Fig. 2. CE–MALDI-MS of a mixture of six peptides. (a) CE separation of six peptides (angiotensin, bradykinin, Arg^8-vasopressin, LHRH, oxytocin and sleep-inducing peptide) on an underivatized fused-silica capillary; (b–d) MALDI spectra of (b) unresolved fraction F1 containing the most basic peptides, bradykinin, angiotensin, Arg^8-vasopressin and LHRH; (c) fraction F2 containing LHRH; (d) fraction F3, oxytocin.

Adsorption of the peptide on the capillary walls cannot be ruled out with non-derivatized fused-silica columns under acidic buffer conditions (acetic acid, pH 3.4).

For the molecular mass determination of proteins by MALDI-MS, it has been important to calibrate the instrument with standard proteins. This has been done by external calibration, or by internal calibration by cocrystallization of matrix with the sample and a standard protein. For external calibration, the mass spectra of the standard protein and the sample must be acquired at the same laser energy. Using low amounts of sample, the laser energy needed to obtain sufficient sensitivity is usually high, resulting in a low mass resolution. Using an internal standard for calibration yielded higher mass accuracy, but was sometimes complicated by suppression of the analyte signals by the standard, due to irregularities in crystal formation or in the desorption process. Care must be taken to add the correct amount of internal standard, so that suppression of the analyte does not occur. Because the sample amounts isolated from CE were in the sub-picomole level, these problems must be considered when calibrating the instrument. Examples of CE–MALDI-MS, with internal and external calibration, are shown in Figs. 3–6. The molecular mass determinations from these CE–MALDI-MS experiments are summarized in Table 3.

Apolipoproteins tend to aggregate due to their amphipathic character [15]. For the CE separation of apolipoproteins, the addition of detergents has, therefore, been used with non-derivatized fused-silica capillaries [16] which minimized aggregation of proteins and capillary

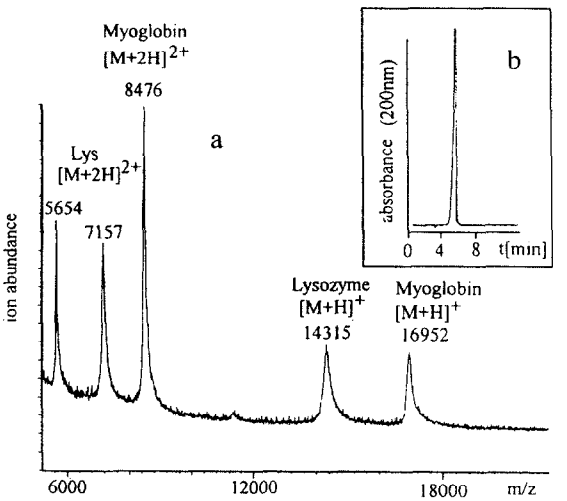

Fig. 3. APS CE–MALDI-MS of hen eggwhite lysozyme. (a) APS CE separation; (b) MALDI-MS with 1 pmol of myoglobin (M_r 16 951) spiked onto the target as the internal standard. M_r (hen eggwhite lysozyme) determined from CE–MALDI: 14 314, theoretical M_r = 14 306.

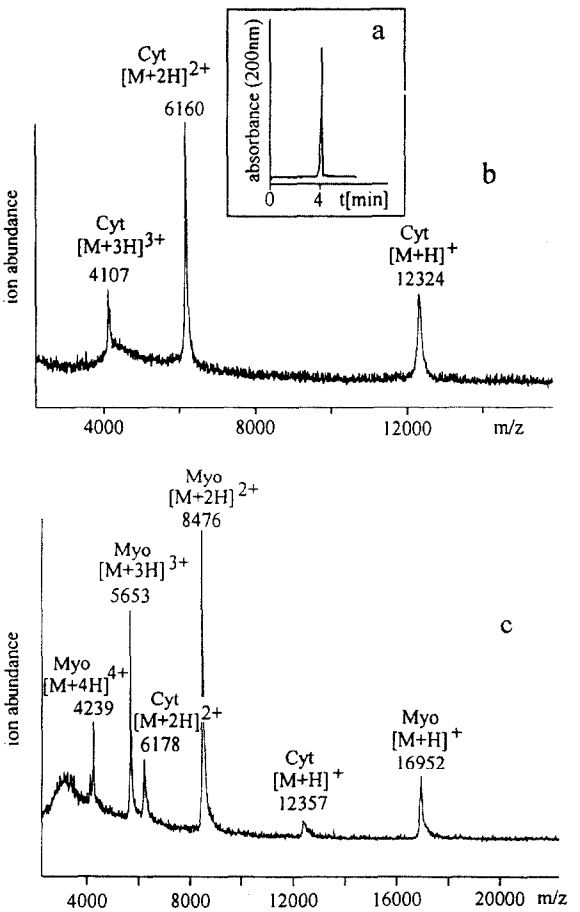

Fig. 4. APS CE–MALDI-MS of cytochrome *c* (Cyt). (a) APS CE separation; (b) MALDI-MS after CE isolation and transfer using an external calibrant; (c) MALDI-MS on the same target as in (b) with 1 pmol of myoglobin (Myo, M_r 16 951) spiked onto the target as the internal standard. M_r determined from CE–MALDI with external calibration (b): 12 322 and with internal calibration (c) 12 357 (theoretical M_r 12 359 D).

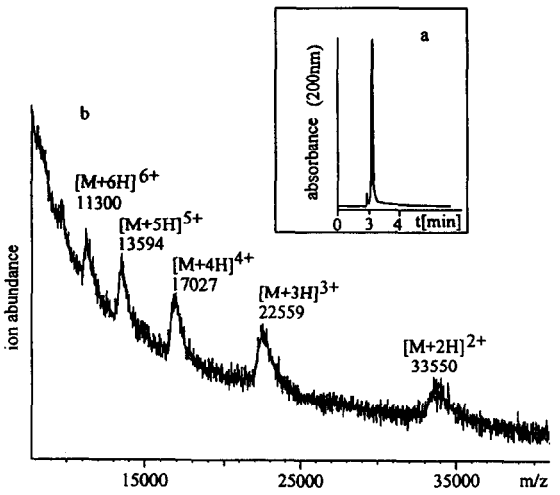

Fig. 5. APS CE–MALDI-MS of bovine serum albumin. (a) APS CE separation, (b) MALDI-MS. M_r determined with external calibration 67 727 (theoretical M_r 66 430). Molecular mass shift is attributable to a higher laser energy being used for the acquisition of the bovine serum albumin spectrum. Addition of 0.2 pmol myoglobin onto the target as internal standard suppressed all analyte signals.

wall adsorption. To avoid interference of detergents from the CE separation with the subsequent MALDI-MS analysis an ApoAII sample [17] was examined on APS CE capillaries using a 10 m*M* acetic acid buffer without addition of detergent. The use of detergent was avoided to prevent any sample suppression and/or peak-broadening during the MALDI-MS analyses. The separation of at least three components in the protein mixture could be obtained by APS CE. On normal fused-silica capillaries, the expected elution order is oligomer, dimer and then monomer due to the higher charge density of the dimer and oligomer [18,19]. The migration order is reversed, however, on the APS-derivatized capillaries, because migration now is from a high negative potential to ground. The characterization by MALDI-MS and the characterization of the reduced protein by CE showed that the major components are ApoAII monomer, ApoAII dimer and non-covalently bound ApoAII oligomers (Fig. 6). Because the doubly protonated homodimer and the singly protonated monomer yield identical *m/z* values, the differences in the two electropherograms were

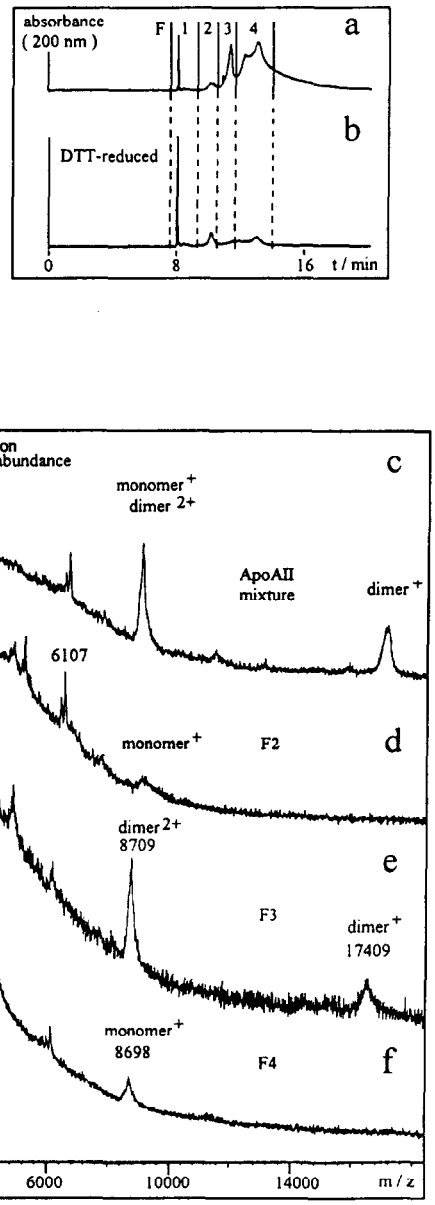

Fig. 6. APS CE and CE–MALDI-MS of apolipoprotein AII. (a) APS CE of Sigma ApoAII, fractions F1, F2, F3 and F4 were collected; (b) APS-CE of ApoAII after disulfide reduction; (c) MALDI-MS of unseparated ApoAII showing two main components with molecular masses of 8700 and 17 400 (the ion at 6107 is background); (d) MALDI-MS of fraction F2 showing a trace of monomer; (e) MALDI-MS of fraction F3, Apo AII homodimer, singly protonated (*m/z* 17 409) and doubly protonated (*m/z* 8709); (f) MALDI-MS of fraction F4, ApoAII monomer (*m/z* 8698) arising from non-covalent oligomers. No ions were observed in the MALDI spectrum of fraction F1 which contained sample buffer salts.

Table 3
Molecular mass determination of proteins by CE–MALDI-MS using internal or external standard

Protein	Theoretical M_r	M_r determined by CE–MALDI-MS	Type of calibration (with myoglobin)	Error (%)
Lysozyme	14 306	14 313	Internal	0.05
Cytochrome *c*	12 359	12 322	External	0.3
		12 357	Internal	0.02
ApoAII monomer	8 690	8 697	External	0.08
ApoAII dimer	17 378	17 408	External	0.2
Bovine serum albumin	66 430	67 727	External	2

important to show the absence of the disulfide-bound homodimer (F3) in the reduced protein. A comparative analysis of the ApoAII protein preparation by PAGE showed the presence of small amounts of ApoAII monomer in addition to the homodimer and, after reduction, only the monomer could be found (see Experimental). The broad peak in the electropherogram (F4) is attributed to non-covalent oligomers of ApoAII. Non-covalent complexes are typically broken up upon target preparation, and, thus, show only ions due to the monomer.

4. Discussion

Using a "coaxial sheath-flow" interface, high sheath-flow-rates of 20 μl/min for fraction collection and a second step of sample preparation, lyophilization and redissolution in matrix solution, the combination of CE and MALDI-MS can be used for the separation and mass spectral identification of peptides and proteins. Compared to other CE–MS interfacing techniques, the main advantage of this "off-line" CE–MALDI technique is the possibility of MS data acquisition times longer than the peak elution time, which increased MS sensitivity. Mass calibration is not easy and has to be determined for each sample. The mass resolution of MALDI-MS, which is strongly influenced by the presence of alkali salts, can be increased by using APS-derivatized CE capillaries with volatile buffers such as acetic acid, and avoiding the addition of alkali ions. The CE peak broadening of apolipoproteins due to aggregation cannot be avoided in the APS CE separation. The addition of detergents (*e.g.* SDS which contains sodium ions!) to CE–MALDI, using low amounts of proteins (low picomole level) might be detrimental to MALDI-MS and has to be examined separately.

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