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Analytical development of electrospray and nanoelectrospray mass spectrometry in combination with liquid chromatography for the characterization of proteins

Thilo A. Fligge, Kai Bruns, Michael Przybylski*

Fakultät für Chemie, Universität Konstanz, Fach M731, 78457 Konstanz, Germany

Abstract

Mass spectrometry has significantly extended its applicability in the area of characterization of protein structures. Electrospray ionization enables on-line coupling with liquid chromatography which has become a powerful tool for the characterization of peptide and protein mixtures. The most recent development of a nanoelectrospray source, using capillary forces for a particularly mild analyte transport and ionization into the mass spectrometer, opens a wide field for applications to protein structure analysis. In this paper, the analytical development of liquid chromatography–electrospray ionization mass spectrometry and nano-electrospray ionization mass spectrometry, adapted to an electrospray ionization quadrupole mass spectrometer and its application to the characterization of noncovalent protein complexes are described. © 1998 Elsevier Science B.V.

Keywords: Mass spectrometry; Electrospray ionization; Nanoelectrospray ionization; Proteins; Polypeptides

1. Introduction

In the last few years mass spectrometry (MS) has made a breakthrough in its applicability to the direct characterization of macromolecules. In particular, intact molecular ions of biopolymers such as proteins and polynucleotides have been obtained due to the development of the ''soft'' desorption–ionization techniques, matrix-assisted laser desorption (MALDI) and electrospray ionization (ESI)-MS [1,2]. Proteins and nucleic acids with molecular masses higher than 100 000 Dalton (Da) have become amenable to MALDI-MS and ESI-MS [3,4]. Despite the relatively high stability to direct fragmentation of macromolecular ions, soft ionization techniques have already found successful applications to primary structure analysis. In combination with specific chemical (e.g. enzymatic) degradation reactions, sequence determinations and the identification of covalent posttranslational structure modifications of polypeptides and proteins have been demonstrated [5]. The direct MS analysis of complex proteolytic peptide mixtures (peptide mapping) has become a powerful tool for the identification and characterization of peptides and proteins, in conjunction with protein sequence data bases [6]. Moreover, several applications to structure–function studies of proteins have been reported recently, such as the determination of epitope sequences in antigen–antibody complexes [7,8], and the characterization of isoenzymes [9] and recombinant proteins [10].

These application perspectives have been considerably extended by the development of ESI-MS,

*Corresponding author. Tel.: +49 7531 882497; fax: +49 7531 883097; e-mail: Michael.Przybylski@uni-konstanz.de

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providing data about protein structure in solution obtained from multiply charged molecular ion distributions [11]. A considerable number of studies by ESI-MS in the last few years have demonstrated successful identifications of specific, noncovalent supramolecular complexes of biopolymers from near-physiological solution conditions [12]. In this article the analytical development of ESI-MS in combination with high-performance liquid chromatography (HPLC) for different bioanalytical applications will be discussed. Unlike other characterization methods, ESI-MS has been shown to be applicable to samples of relatively low purity, and to multicomponent mixtures due to its compatibility with microseparation techniques. In the present study, the performance of a combination of capillary liquid chromatography (LC) with on-line UV and ESI-MS is reported, using a commercial quadrupole mass analyzer for separation and characterization of peptide and protein mixtures. The most recent development of a nanoelectrospray-ionization source (nano-ESI) has been shown to be capable of coping with variation of important solution parameters, while taking advantage of the long spraying time and considerably reduced sample consumption [13–15].

ESI is a desorption method capable of emitting ions from liquid surfaces into the gas phase. A sample solution is pumped at low flow-rates, typically 1–10 μl/min through a fused-silica capillary fixed to a stainless steel needle. Charged aerosol droplets are electrosprayed by means of a strong electric potential between the needle tip and a cylindrical counterelectrode (nozzle) and dispersed into the ionization region [11]. The formed microdroplets are rapidly reduced to small solvated macroions [16,17] influenced by analyte charge, temperature and solvent. Another important parameter for the desolvation of macroions is the small potential difference between the nozzle and a second skimmer-type repeller electrode, referred to as declustering potential, Δ*CS* [11]. Unlike other desorption methods ESI-MS forms multiply charged ions (cations, anions) from peptides and proteins via multiple (de)protonation. The number of charged groups in solution determines the maximum charge numbers of macromolecular ions [11,18]. Initial ESI-MS studies of biopolymers were mainly carried out with acidic solutions [11], in which the number of basic residues shows a rough correlation with the maximum charge state. In addition to temperature and solvent, the declustering potential, Δ*CS*, also has a strong influence on the charge structure of ions. As an example, ESI spectra of horse heart cytochrome C at different declustering potential are compared in Fig. 1. The spectra show a shift of the molecular ion charge distributions to lower charge states at stronger desolvation conditions (increasing Δ*CS*). This effect has been explained by dissociation of protons at the stronger desolvation conditions due to collisions with surrounding molecules [19]. Although electrospray is a mild ionization technique, suitable for the analysis of intact noncovalent complexes [12], the capability to selectively fragment covalent bonds has been shown to be an efficient tool providing structural information [19,20]. Furthermore, fragmentation in the nozzle–skimmer–collimator region due to collision with surrounding molecules depending on the applied declustering potential has been observed [21]. Whereas large biomacromolecules are able to absorb this energy via vibrational relaxation, small

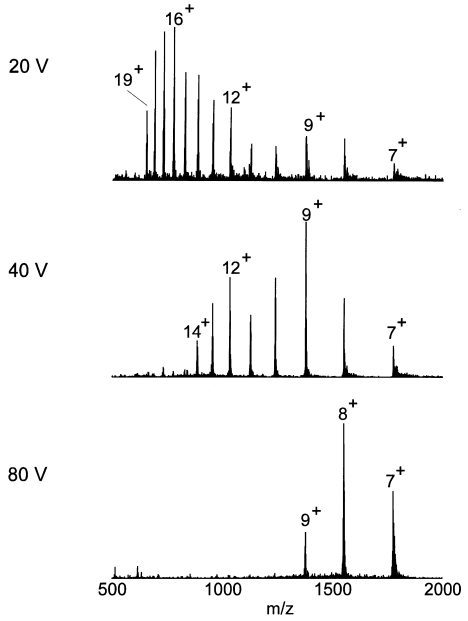

Fig. 1. ESI mass spectra of horse heart cytochrome C (4 μ*M*) at different declustering potential Δ*CS* (20 V, 40 V, 80 V). The solvent used was a 95:5 mixture of aqueous acetic acid (2%) and methanol. The molecular mass observed was $M_{r,exp}$ 12 360 Da (M_r 12 360 Da).

peptides undergo fragmentation which for small peptides provides sequence information by changing the ΔCS. However, in ESI mass spectra of peptide mixtures this feature can lead to increasingly complex ion patterns, thus requiring additional methods for resolution. Recently, a new ESI source has been developed following a theoretical investigation into the electrospray mechanism [13,22]. The nano-ESI ion source differs from conventional electrospray sources by its solvent delivery mechanism which provides several analytical advantages. The ion-formation model predicts a dependence between the droplet size emitted from the tip of the Taylor cone, and the flow-rate used. Lower flow-rates lead to smaller droplets with favourable analytical properties. For example, the enhanced surface-to-volume ratio makes a large proportion of analyte molecules accessible to desorption and ionization. Additionally, low flow-rates provide long measurement times enabling more extensive sample investigations. The utility of the nano-ESI technique, adapted to a conventional ESI source [14,15], has been examined in this study for the characterization of proteins, including the direct analysis of noncovalent supramolecular complexes.

2. Experimental

2.1. Proteins and polypeptides

The following commercially available peptides and proteins were used: cytochrome C, hen eggwhite lysozyme, horse heart myoglobin, bovine serum albumin, luteinizing hormone-releasing hormone (LHRH) (acetate), melittin, angiotensin II from Sigma (St. Louis, MO, USA) and LWMRFA from Serva (Heidelberg, Germany). Homogeneity was ascertained by standard HPLC techniques and by MS (MALDI-MS).

2.2. ESI-MS

ESI mass spectra were obtained with a Vestec A201 single-quadrupole mass spectrometer (Vestec, Houston, TX, USA), fitted with a 10 kV conversion dynode and a 2000 m/z mass range, as described in Ref. [19]. For conventional ESI investigations, samples were infused by a syringe pump (Harvard Apparatus, South Natick, MA, USA) at a flow-rate of 1 μl/min.

2.3. LC–ESI-MS

A Waters 600E pump was used as the solvent delivery system. The flow-rate was reduced by a precolumn split system (Acurate; LC packings, Amsterdam, Netherlands) which directs a flow of approximately 4 μl/min to the capillary C_{18}-HPLC column (Fusica II, LC packings; C_{18}; 3 μm; 300 Å; 150 mm×0.3 mm I.D.). The chromatograph was interfaced to a modified ABI 759A UV absorbance detector (Applied Biosystems, Weiterstadt, Germany) equipped with a U-shaped flow cell (LC packings). UV absorbance detection at 214 nm was used additionally for monitoring the peptide elution, thus aiding the determination of the area of the chromatogram suitable for obtaining optimal MS information. The LC effluent was connected via a fused-silica capillary directly to the electrospray interface. Solvents used in reversed-phase HPLC usually consist of aqueous mixtures of acetonitrile with less than 0.1% of an ion pairing reagent. Taking advantage of the full compatibility of ESI-MS with this mobile phase no further modification of the solvent is required. Furthermore, trifluoroacetic acid (TFA) is a widely used mobile phase modifier for biological samples. Due to its acidity TFA increases the conductivity of water, so that the high conductivity and high surface tension can cause problems for the ESI process [23]. The resulting instability may lead to a breakdown of the spray and reduction of sensitivity [24], hence particular care has been taken to manually adjust the needle voltage and maintain a constant spray during changes of solvent composition in gradient separations.

2.4. Nano-ESI-MS

For nano-ESI investigations, the Vestec ion source was replaced by the nano-ESI source shown in Fig. 2. Borosilicate glass capillaries of the type GC120F-10 from Clark Electromedical Instruments (Pangbourne, UK) were used for manufacturing the microcapillaries which were pulled with a capillary puller model P-97 from Sutter Instruments (Novato,

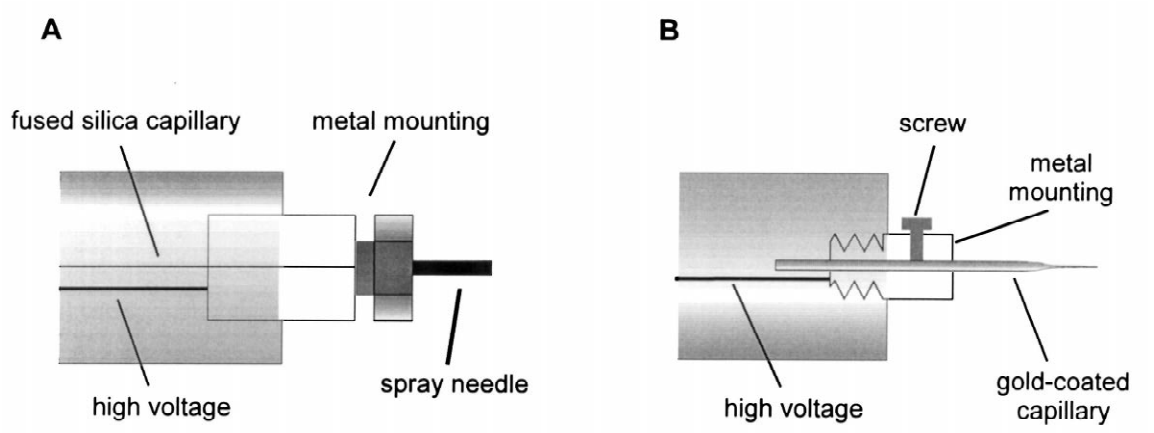

Fig. 2. Comparison of (A) the conventional needle tip and electrospray interface, and (B) the nanoelectrospray interface. A gold-coated borosilicate capillary is fixed via a PTFE screw to a metal mounting at which the high voltage (1–2 kV) is applied.

CA, USA) in a two-step cycle as described elsewhere [14]. The capillaries were then coated with a thin layer of gold by a International Scientific Instrument sputter for providing the high voltage connection. A sample of 0.5–2 μl was injected by gel-loader tips (Eppendorf, Hamburg, Germany), or by dipping the capillary into the sample solution. In case of plugging, the capillary was reopened by briefly touching it against a glass plate. The loaded capillary was fixed via a PTFE screw in a metal mounting and then pushed forward into the ion source region. The voltage at the capillary tip was manually adjusted to 1.1–1.3 kV, with a declustering potential typically set to 20 V. In order to obtain the most stable spray the capillary has to be manually adjusted to the nozzle of the ion source.

The nanoelectrospray has been found to be stable at flow-rates of 20–40 nl/min which appears to be lower than attainable by a pumped system. The minimum flow-rate used for conventional electrospray is 1–5 μl/min, hence sample consumption is drastically decreased by the nanoelectrospray source.

3. Results and discussion

The combination of ESI-MS with LC separation has emerged as a powerful tool for the separation and structure characterization of, e.g., complex mixtures of biological origin [25,26]. Yamashita and Fenn first demonstrated the performance of LC–MS using an ESI interface [27]. Further applications have demonstrated the possibility of using a pneumatic nebulization interface allowing ESI of LC effluents up to 500 μl/min [28] for the analysis of protein and peptide mixtures [29]. The introduction of microcolumn-LC has provided the possibility for direct combination with both ESI as well as fast atom bombardment (FAB) for generating gas-phase ions from biomolecules in solution [30–32]. Due to several problems concerning the viscous matrix system and the limited mass-to-charge range of FAB-MS the combination LC–ESI-MS has developed as a more efficient technique for the analysis of peptides and proteins [33,34].

The performance of this LC–ESI-MS combination was tested in the analysis of a mixture of the three model peptides, LHRH (1183 Da), angiotensin II (1046 Da) and melittin (2847 Da). The chromatographic profiles obtained and ESI mass spectra of the different chromatographic peaks are shown in Fig. 3. The direct ESI mass spectral analysis of an equimolar mixture of the peptides showed a multiplicity of singly to quadruply protonated molecular ions which illustrate the problems arising in a complete identification (data not shown). The LC–ESI-MS chromatographic profile showed, besides three abundant components (1–3), two additional peaks (4,5) at higher retention times which were identified by ESI-MS and presumably caused by contamination of the melittin sample. The profile of the total ion current in ESI-MS is complementary in principle to the chromato-

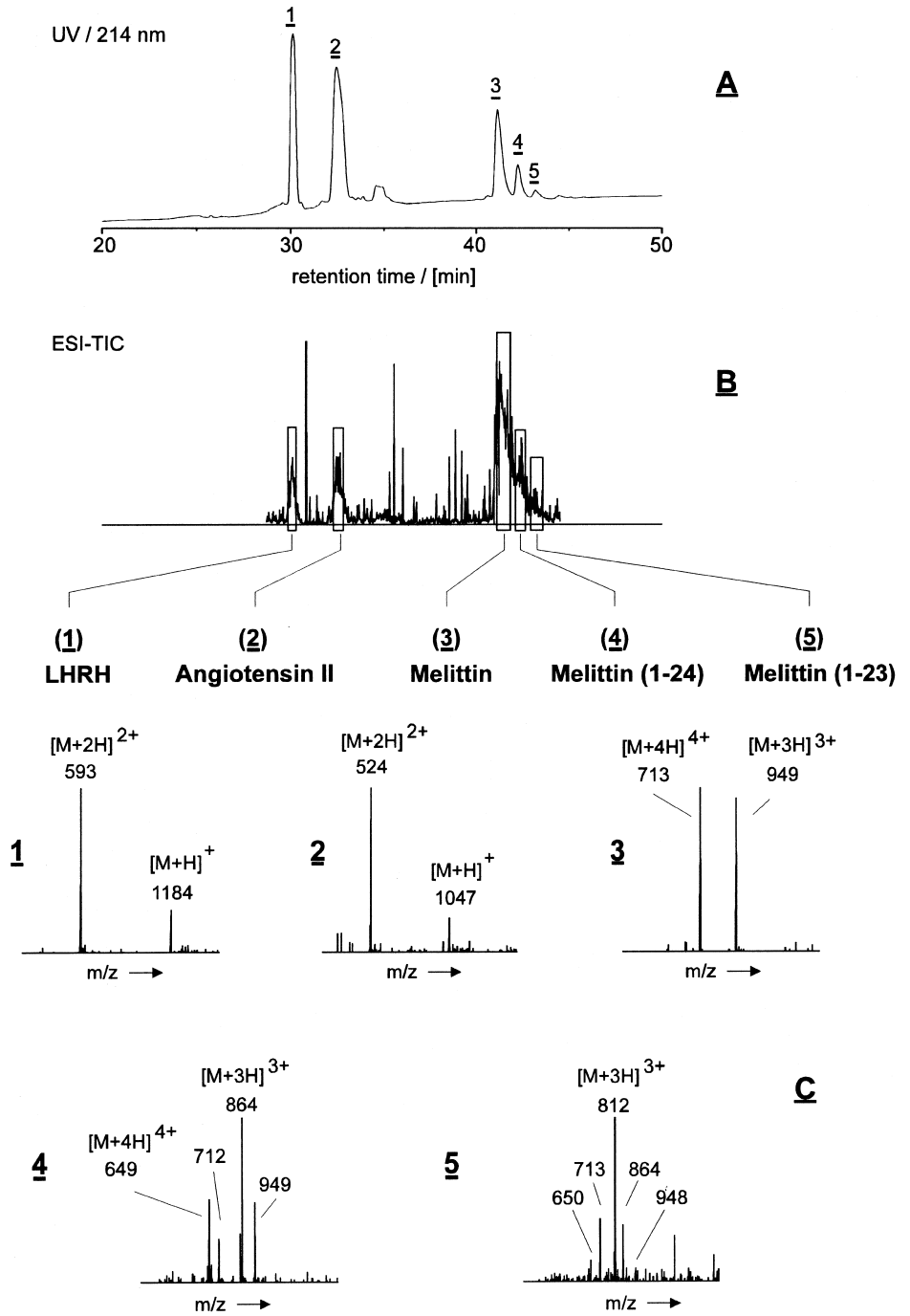

Fig. 3. Separation and characterization of a peptide mixture by LC–ESI-MS. (A) UV detection profile; (B) total ion current (TIC) trace of ESI-MS following an injection of an equimolar mixture of luteinizing hormone-releasing hormone (LHRH), angiotensin II and melittin (100 pmol each). Chromatographic conditions were: eluent A=water–0.1% TFA and eluent B=acetonitrile–0.1% TFA using a linear gradient from 10% B to 90% B over 40 min. The mass spectrometer was scanned from m/z 300–1800 with an acquisition rate of 2 s/scan. Scans used for generating the mass spectra in (C) are boxed. Signals denoted are the protonated molecular ions of the corresponding peptide.

graphic profile obtained by UV detection. Thus, the high intensity of peak 3 observed by ESI-MS compared to the UV detection indicates a better ionization of this peptide relative to the other components. The mass spectral data of the separate chromatographic peaks enables the assignment of molecular ions for each peptide, and even leads to the identification of the less abundant peaks 4 and 5. Due to the singly and doubly protonated molecular ions peaks 1 and 2 can be assigned to LHRH and angiotensin II, respectively. The mass spectrum obtained for peak 3 showed signals at m/z 713 and 949 due to the $[M+4H]^{4+}$ and $[M+3H]^{3+}$ ions of full-length melittin (1–26). Due to band broadening and tailing effects the mass spectrum of the chromatographic peak 4 showed molecular ions of intact melittin, and additional peaks at m/z 649 and 864 which can be assigned to $[M+4H]^{4+}$ and $[M+3H]^{3+}$ of a carboxy-terminal shortened melittin (1–24). The even smaller chromatographic peak 5 leads to a mass spectrum containing signals from the peaks 3 and 4 but also a signal at m/z 812 that can be assigned to $[M+3H]^{3+}$ of melittin (1–23). A summary of the data for the identified components is shown in Table 1. The analytical utility of the LC–ESI-MS combination is reflected by an increasing number of applications over the last few years in fields such as environmental sciences [35], drug analysis [36], and biochemistry [33,37].

Several factors may contribute to the high efficiency of desolvation and ionization of analyte molecules in the nanoelectrospray source [13]. The analyte molecules are suggested to be separated into different droplets preventing their clustering. Thus, suppression effects caused by sample components such as buffer or detergents which may be due to charge competition among mixture components are greatly reduced. Fig. 4 illustrates that ESI spectra obtained by nanoelectrospray and conventional electrospray are qualitatively comparable. No significant decrease of signal-to-noise ratio or signal intensities was observed. A slight shift of the charge distribution to lower protonated molecular ions was observed for both hen eggwhite lysozyme and the model peptide LWMRFA. A comparison of the mass spectral information obtained for different peptides and proteins is summarized in Table 2. The deviation in mass accuracy for the bovine serum albumin obtained by the nano-ESI and the conventional ESI-MS may possibly result from a lower adduct formation in the nano-ESI analysis. These results illustrate that nano-ESI provides similar data in principle, while consuming ca. 100 times less sample amount than conventional ESI. The nanoelectrospray interface disperses the liquid sample exclusively by electrostatic forces without the need of sheath flow, nebulizing gas or air pressure. Nevertheless, the present source fitted to a Vestec ESI interface enabled a stable electrospray process even when using a wide variety of buffers, detergents or chaotropic reagents [15]. Model studies with hen eggwhite lysozyme using urea and detergent containing solutions with high concentrations provided a high sensitivity of the developed nano-ESI source (Fig. 5). In both cases, the protonated molecular ions of lysozyme were detected as abundant peaks. In case of the *n*-octylglucoside containing sample, a shift to higher charged molecular ions was observed. Although cluster ions of urea and *n*-octylglucoside were detected, no adduct formation or loss in signal-to-noise ratio was observed.

Due to its high tolerance to buffer systems, nearly physiological conditions can be applied by nano-ESI. This feature becomes very interesting in the analysis

Table 1
Sequence and molecular masses of peptides observed in the LC–ESI-MS analysis of the peptide mixture, luteinizing hormone-releasing hormone (LHRH), angiotensin II, and melittin

Peak	Peptide	Sequence	Molecular mass (Da)	Molecular ions obtained
1	LHRH	Pyr-HWSY GLRP	1183.3	$[M+H]^{+}$, $[M+2H]^{2+}$
2	Angiotensin II	DRVYI HPF	1046.2	$[M+H]^{+}$, $[M+2H]^{2+}$
3	Melittin (1–26)	GIGAV LKVLT TGLPA LISWI KRKRQ Q	2847.5	$[M+3H]^{3+}$, $[M+4H]^{4+}$
4	Melittin (1–24)	GIGAV LKVLT TGLPA LISWI KRKR	2591.2	$[M+3H]^{3+}$, $[M+4H]^{4+}$
5	Melittin (1–23)	GIGAV LKVLT TGLPA LISWI KRK	2435.1	$[M+3H]^{3+}$

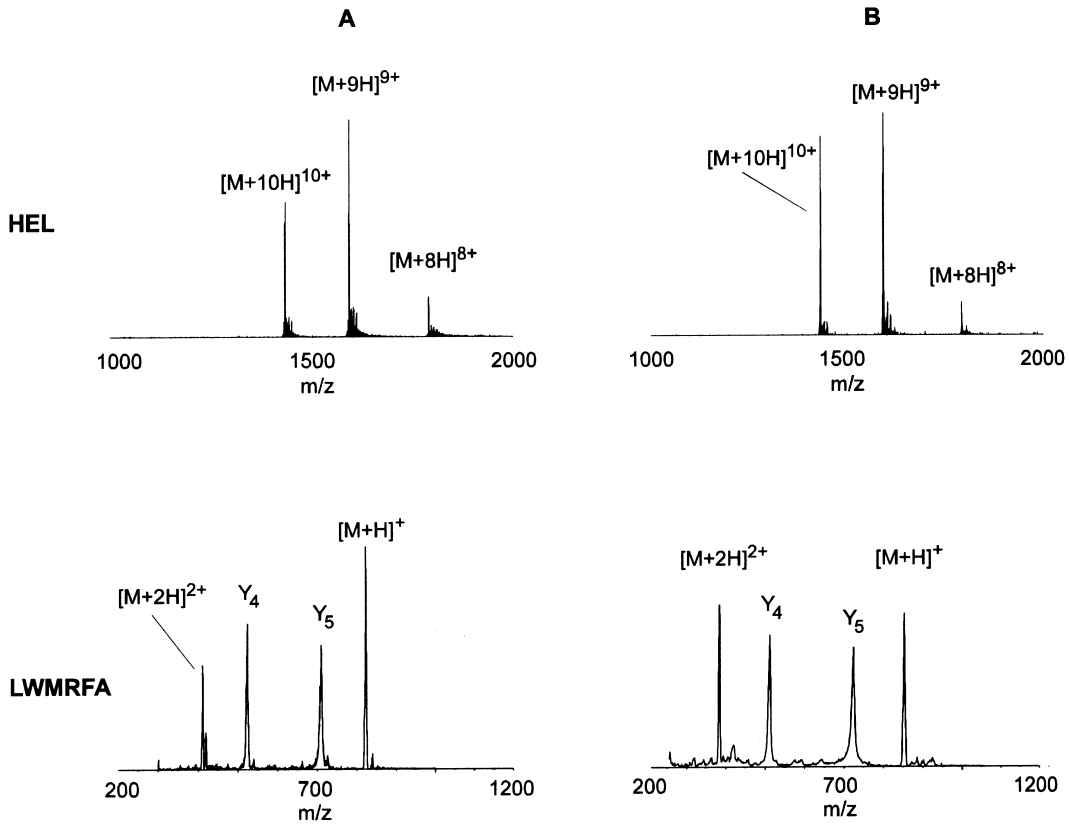

Fig. 4. Comparison of the electrospray mass spectra of hen eggwhite lysozyme (1 μ*M*) and the peptide LWMRFA (60 μ*M*) obtained by (A) nano-ESI, (B) the conventional ESI system. The solvent used was a 95:5 mixture of aqueous acetic acid (2%) and methanol. The declustering potential in each case was held at 20 V.

of noncovalent interactions where the buffer composition is frequently dictated by the need for maintaining an intact supramolecular complex in solution. Furthermore, the possibility of enhanced measurement time and less sample consumption enables extensive studies with regard to solvent composition, temperature effects or influence of pH. The characterization of noncovalent biomolecular complexes by ESI-MS has been recently reviewed [12]. The nano-ESI source significantly enhances the possibilities for analysis of such interactions. An illustrative example is the spectrum of the heme-protein complex myoglobin (Fig. 6), one of the first noncovalent complexes studied by ESI-MS [11,38]. Using ammonium acetate buffer at pH 5, a partial dissociation of the complex leads to the doublet series of multiply protonated molecular ions of the intact heme-protein and the apoprotein, respectively. This result is in agreement with previous studies by conventional ESI-MS [12].

4. Conclusions and perspectives

Using the on-line coupling of LC and ESI, an effective analytical tool for the characterization of complex sample mixtures is provided. As reflected by the number of applications, the analytical utility of the LC–ESI-MS combination, especially in the fields of biological samples, has been investigated. Recent studies have shown the feasibility of miniaturization by the means of a reduced sample consumption. Furthermore, much progress has been made by off-line combination of LC with MS methods such as MALDI-MS and ESI-MS, leading

Table 2
Comparison of ESI mass spectra of peptides and proteins obtained by nano-ESI-MS and conventional ESI-MS, respectively

Peptide/protein (M_r, Da)	Mass spectral parameters	Nano-ESI-MS	Conventional ESI-MS
LWMRFA (823.7)	$M^{b}_{r(exp)}$	823.9	823.8
	Charge distribution[c]	1+–2+	1+–2+
	Most abundant ion[d]	1+	2+
	Fragmentation[e]	Y_4, Y_5	Y_4, Y_5
	Sample amount[f]	12	1800
Lysozyme (14306.5)	$M^{b}_{r(exp)}$	14308	14307
	Charge distribution[c]	8+–10+	8+–10+
	Most abundant ion[d]	9+	9+
	Sample amount[f]	0.07	10
Myoglobin (16950.4)[a]	$M^{b}_{r(exp)}$	16953	16955
	Charge distribution[c]	10+–19+	11+–20+
	Most abundant ion[d]	15+	16+
	Sample amount[f]	3.5	350
Bovine serum albumin (66430)	$M^{b}_{r(exp)}$	66623	66694
	Charge distribution[c]	34+–56+	34+–57+
	Most abundant ion[d]	41+	45+
	Sample amount[f]	0.3	90

[a] Apoprotein; [b] average molecular mass determined from $[M+nH]^{n+}$ ions; [c] minimum and maximum charge states of $[M+nH]^{n+}$ ions observed; [d] most abundant charge state of the $[M+nH]^{n+}$ ion; [e] most abundant fragment ions observed; [f] sample amount (pmol) employed.

to a rapid and more automated sample throughput [39,40].

Taking advantage of the long spraying time, extensive MS investigations with 1 μl sample volumes are provided by the nano-ESI source. This feature enabled successful high sensitivity analyses by several laboratories as reflected by an increasing number of recent publications. The applicability to MS–MS investigations and hence the possibility for the characterization of gel-isolated proteins or even gel-digested mixtures make the nano-ESI source an interesting tool for rapid and sensitive analytical strategies [41–44]. One important feature of nano-ESI is the high tolerance towards buffer composition and detergents. Thus, in the characterization of noncovalent interactions the nano-ESI source can be

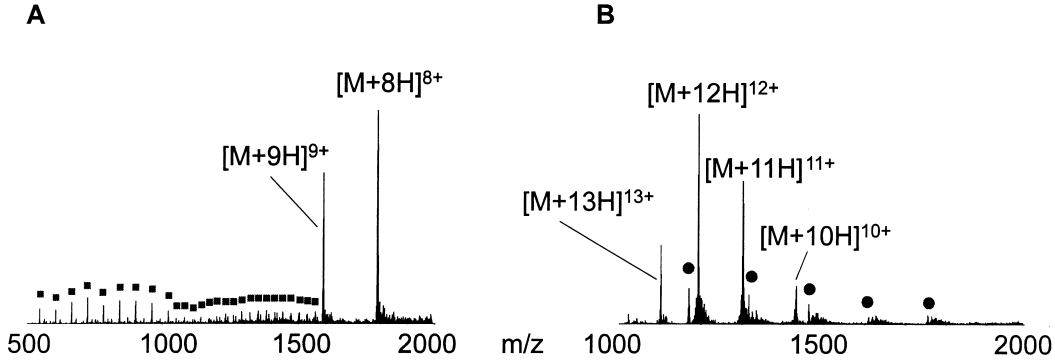

Fig. 5. Nano-ESI mass spectra of hen eggwhite lysozyme (1 μ*M*) in 2% acetic acid:methanol (9:1 v/v) containing (A) 0.5 *M* urea and (B) 14.5 m*M* *n*-octylglucoside. The boxed signals (■) denote signals of urea clusters, the circled signals (●) *n*-octylglucoside clusters, respectively. The molecular mass observed was for (A) $M_{r,exp}$ 14 308 Da and for (B) $M_{r,exp}$ 14 307 Da (M_r 14 306.5 Da).

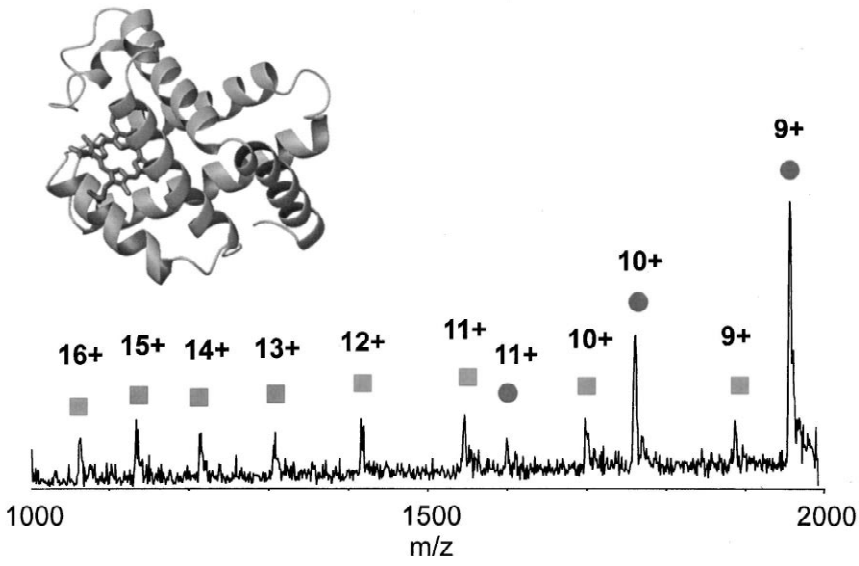

Fig. 6. Nano-ESI mass spectrum of the heme-protein complex of horse-heart myoglobin (3 μ*M*). The spectrum was obtained in 5 m*M* ammonium acetate:methanol (95:5; pH 5) with a declustering potential of 20 V. The circled signals (●) denote heme-protein ions, the boxed signals (■) the apoprotein ions, respectively. The molecular mass observed was for the apo-myoglobin $M_{r,exp}$ 16 953 Da (M_r 16 950 Da) and for the heme-protein $M_{r,exp}$ 17 569 Da (M_r 17 567 Da). The myoglobin structure was redrawn from the Brookhaven National Laboratory data base, prepared using MOLMOL [47].

expected to be an efficient tool for the MS characterization of protein–protein interactions, metal–ion complexes and antigen–antibody interactions [8,45,46].

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