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Sensitivity considerations for large molecule detection by capillary electrophoresis–electrospray ionization mass spectrometry

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ABSTRACT

The use of the electrospray ionization (ESI) method for interfacing capillary electrophoresis with mass spectrometry (CE–MS) is particularly well suited for the analysis of large molecules due to the multiple charging phenomenon. While ionization efficiency is very high, the available ion current is dispersed over more peaks so that the maximum peak intensity obtainable declines significantly for large molecules. Sensitivity with ESI can be improved by operation at very low flow-rates, an ideal situation for CE–MS. These and other considerations related to sensitivity are illustrated using ESI–MS measurements for cytochrome *c*.

INTRODUCTION

Capillary electrophoresis (CE) in its various manifestations (free solution, isotachophoresis, isoelectric focusing, polyacrylamide gel, micellar electrokinetic "chromatography") is attracting growing attention as a method for rapid high resolution separations of very small sample volumes of complex mixtures. In combination with the sensitivity and selectivity of mass spectrometry (MS), CE–MS becomes a potentially powerful bioanalytical technique[1–12]. The first CE–MS combination was based upon the electrospray ionization (ESI) approach[1–10]. More recently continuous-flow fast atom bombardment interfaces have also been demonstrated[11,12]. The ESI method has recently been demonstrated (off-line) for biomolecules exceeding 100 000 dalton[4]. The method provides extremely high ionization efficiencies [*i.e.*, very high (molecular ions produced)/(molecules consumed)] and precise molecular mass (M_r) measurements[4]. For the combined CE–MS of large biomolecules detection sensitivity becomes the overriding factor, often determining the success or failure of a particular electrophoretic method, buffer system, or application.

In this report, we consider the important subject of sensitivity, which defines the

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ultimate potential of the CE–ESI-MS technique. Sensitivity is considered from the viewpoints of sample size, concentration and flow-rate, overall detection efficiency [*i.e.*, (ions detected)/(molecules introduced)], and actual ionization efficiency. The high charge state molecular ions produced by ESI allow tandem mass spectrometry to be extended to much higher M_r than previously possible and places even greater demands upon the ionization method and instrumental performance. The development of combined CE–MS will be crucial for possible extension of ESI to the attomole (10^{-18} M) sample range.

EXPERIMENTAL

The ESI-MS instrumentation used in this study and typical operating conditions have been previously described[4,8,9]. Cytochrome *c* in aqueous acidic buffer solutions, consisting of 5% glacial acetic acid, were introduced to the ESI source through a 100 μm I.D. fused-silica capillary at a rate of 1 μl/min. The flow mixes with a liquid sheath electrode, typically methanol, flowing at 3 μl/min, at the tip of the electrospray ionization source[1]. Analyte and sheath flow are independently controlled by separate syringe pumps. A potential of +5 kV is applied to the sheath electrode, producing highly charged liquid droplets of *ca.* 1 μm diameter at atmospheric pressure in a flow of dry nitrogen to aid the desolvation process. The ESI source is mounted 1.5 cm from the entrance orifice of the quadrupole MS. Highly charged ions are sampled through a 1-mm nozzle orifice and 2-mm skimmer and are efficiently transported through a cryo-pumped region by the radio-frequency (rf) quadrupole lens to a quadrupole mass spectrometer for detection. For positive ions, the typical focusing lens voltage is +1 kV, with the nozzle at +200 V and the skimmer at ground potential. The mass spectrometer (EXTREL, Pittsburgh, PA, U.S.A.) used for these studies has an effective m/z range of 1700. The study of cytochrome *c* at various concentrations used slow 1.5-min scans to cover the entire m/z range. Peak abundances were collected only at integer m/z values using our current data system (Teknivent, St. Louis, MO, U.S.A.). Routine calibration of the m/z scale for ESI-MS was performed with low-molecular-mass polymers, such as polyethylene glycol (average molecular mass 1000), monitoring both the singly charged (singly sodiated) and doubly charged (doubly sodiated) molecular ion distributions. The horse heart cytochrome *c* was obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification.

RESULTS AND DISCUSSION

The electrospray ionization process

Although the use of ESI for MS is a relatively recent occurrence, it and related phenomena have been extensively investigated[13–24]. Electrospray ion production requires two steps: dispersal of highly charged droplets at near atmospheeric pressure, followed by conditions resulting in droplet evaporation. An electrospray is generally produced by application of a high electric field to a small flow of liquid (generally 1–10 μl/min) from a capillary tube. A potential difference of 3 to 6 kV is typically applied between the capillary and counter electrode located 0.5 to 2 cm away, where ions may be sampleed by the mass spectrometer through a small orifice. The electric field results in charge accumulation on the liquid surface at the capillary terminus; thus

the liquid flow-rate, resistivity, and surface tension are important factors in droplet production. The high electric field results in disruption of the liquid surface and formation of highly charged liquid droplets. Positively or negatively charged droplets can be produced depending upon the capillary bias. (The negative ion mode requires the presence of an electron scavenger such as oxygen to inhibit electrical discharge[25].) While a wide range of liquids can be sprayed electrostatically into vacuum[16], or with the aid of nebulizing gas[15,26], the use of only electric fields leads to some practical restrictions on the range of liquid conductivities and dielectric[18,19]. Solution conductivities of $\lesssim 10^{-5}\ \Omega^{-1}$ are required for a stable electrospray at useful liquid flow-rates[18], corresponding to an aqueous electrolyte solution of $\lesssim 10^{-4}$ *N*. Typical ESI currents for water–methanol–5% acetic acid solution are in the range of 0.1–0.5 μA. In the mode found most useful for ESI-MS, an appropriate liquid flow-rate results in dispersion of the liquid as a fine mist. A short distance from the capillary the droplet diameter is often quite uniform and on the order of 1 μm[21]. Of particular importance is that the total eleectrospray ion current increases only slightly for higher liquid flow-rates. Increasing flow-rates result in formation of larger droplets and ultimately electrical breakdown, although the use of a nebulizing gas can produce stable results at flow-rates as large as 100 μl/min[26]. The use of higher voltages does not substantially increase the electrospray ion current until the onset of a corona discharge (generally at >6 kV).

M_r measurements are obtainable since ESI-mass spectra generally consist of a distribution of molecular ion charge states without contributions due to dissociation. The envelope of charge states, for proteins arising generally from proton attachment, gives a distinctive pattern of peaks due to the quantum nature of electronic charge; *i.e.*, adjacent peaks appear always to vary by addition or subtraction of one charge. A striking feature of electrospray ionization mass spectra of many proteins is that the average charge state increases in an approximately linear fashion with M_r, although this observation is no doubt skewed by the nature of the MS "observational window". As first reported by Fenn and co-workers[27,28] the M_r of a macromolecule may be immediately determined from spectra such as those in Fig. 1.

ESI-MS sensitivity of detection for macromolecules

The efficiency of ESI can be very high, providing the basis for extremely sensitive measurements. As shown in Table I, current instrumental performance can provide a total ion current at the detector of *ca.* $2 \cdot 10^{-12}$ A, or *ca.* 10^{7} counts/s for singly charged species. This is consistent with performance of instruments in our laboratory. For an analyte solution flow-rate of 1 μl/min, a 10^{-4} *M* solution of a singly charged species can account for the total electrospray ion current. At higher concentrations such analytes appear to "saturate" the mass spectrum and displace "normal" solvent-related peaks at low *m/z*. If the analyte carries more than one charge, the concentration which will saturate the ESI process decreases proportionately. On the basis of the instrumental performance given in Table I, concentrations as low as 10^{-10} *M*, or *ca.* 10^{-18} mole/s, of singly charged species can be expected to yield detectable ion currents (*ca.* 10 counts/s) if the analyte is completely ionized. Indeed, we have obtained detection limits of $\ll 10^{-8}$ *M* in characterization of readily ionized ionic species in precipitation samples and low attomole detection limits for quaternary ammonium ions in conjunction with capillary zone electrophoresis[2,3].

TABLE I

TYPICAL ELECTROSPRAY ION SOURCE CHARACTERISTICS

Ionization: total current, 1–5 · 10^{-7} A; unit charges/s, 0.6–3 · 10^{12}; droplet diameter[a], *ca.* 1–2 μm.

	Ion sampling (nozzle–skimmer or capillary inlet–skimmer) efficiency[b]		
	% of total ionization	*Current (A)*	*Total ions/s*[c]
Through nozzle[d]	$\approx 10^{-2}$	$\approx 2 \cdot 10^{-9}$	$\approx 10^{10}$
Focused into quadrupole	$\approx 10^{-4}$	$\approx 2 \cdot 10^{-11}$	$\approx 10^{8}$
Detected[e]	$\approx 10^{-5}$	$\approx 2 \cdot 10^{-12}$	$\approx 10^{7}$

[a] Estimated at *ca.* 0.3 cm from capillary using 10^{-4} *N* electrolyte solution of water–methanol (50:50) solution. Droplets generally become too small to be visible (<0.3 μm) at 0.5 cm from capillary.

[b] Approximate performance measured using a quadrupole mass spectrometer described in ref. 1. We assume 2 · 10^{-7} A total ESI current.

[c] For a singly charged species.

[d] 1-mm-diameter nozzle or a slightly larger capillary bore giving an equivalent gas flow.

[e] At m/z 1000 based upon both direct Faraday cup current measurements and ion counting utilizing an electron multiplier.

Fig. 1 gives ESI-mass spectra obtained for aqueous solutions of horse heart cytochrome *c* (M_r 12 360) by direct infusion at 0.5 to 1 μl/min using the sheath flow ESI interface[1]. Spectra were acquired in 90-s scans for solution concentrations ranging from 1.5 · 10^{-4} *M* (Fig. 1A) to 1.5 · 10^{-8} *M* (Fig. 1F). For the most dilute solution a spectrum of quality adequate for M_r determination required only 23 fmol of cytochrome *c*. The mass spectra are dominated by the distribution of multiply protonated molecular ions, but other contributions are also evident. For example, Fig. 1B shows a scale expansion (×36) for the most concentrated solution (Fig. 1A). The spectrum shows a number of small peaks between m/z 550 and m/z 850 which may be due to small contributions of collisional dissociation in the nozzle–skimmer interface for the most highly charged species[29,30]. For lower charge states small contributions which correspond to multiply charged dimers are also evident.

At cytochrome *c* concentrations below *ca.* 2 · 10^{-5} *M*, two additional observations pertain. First, the relative intensities shifts of higher charge states increase and become nearly independent of solution concentration. Second, the lower charge states show contributions of adduct ions (of 98 $\pm$ 2 a.m.u.) giving a series of small peaks on the high m/z side of each molecular ion. At lower cytochrome *c* concentrations, the relative size of these adduct contributions is nearly constant. This is fortunate since the assumption of molcular ion protonation utilized for M_r determination (although strictly unnecessary[28]) remains valid and unchanged at very low sample concentrations[4,27,28]. Fig. 2 gives the peak intensity of the 10+ to 18+ charge states as a function of analyte flow-rate. Peak intensity increases nearly linearly with sample concentration. Such results suggest that high sample concentrations result in formation of ions *with reduced charge state rather than lower ionization efficiency.* Thus, although the peak intensity for the most abundant charge state is "saturated",

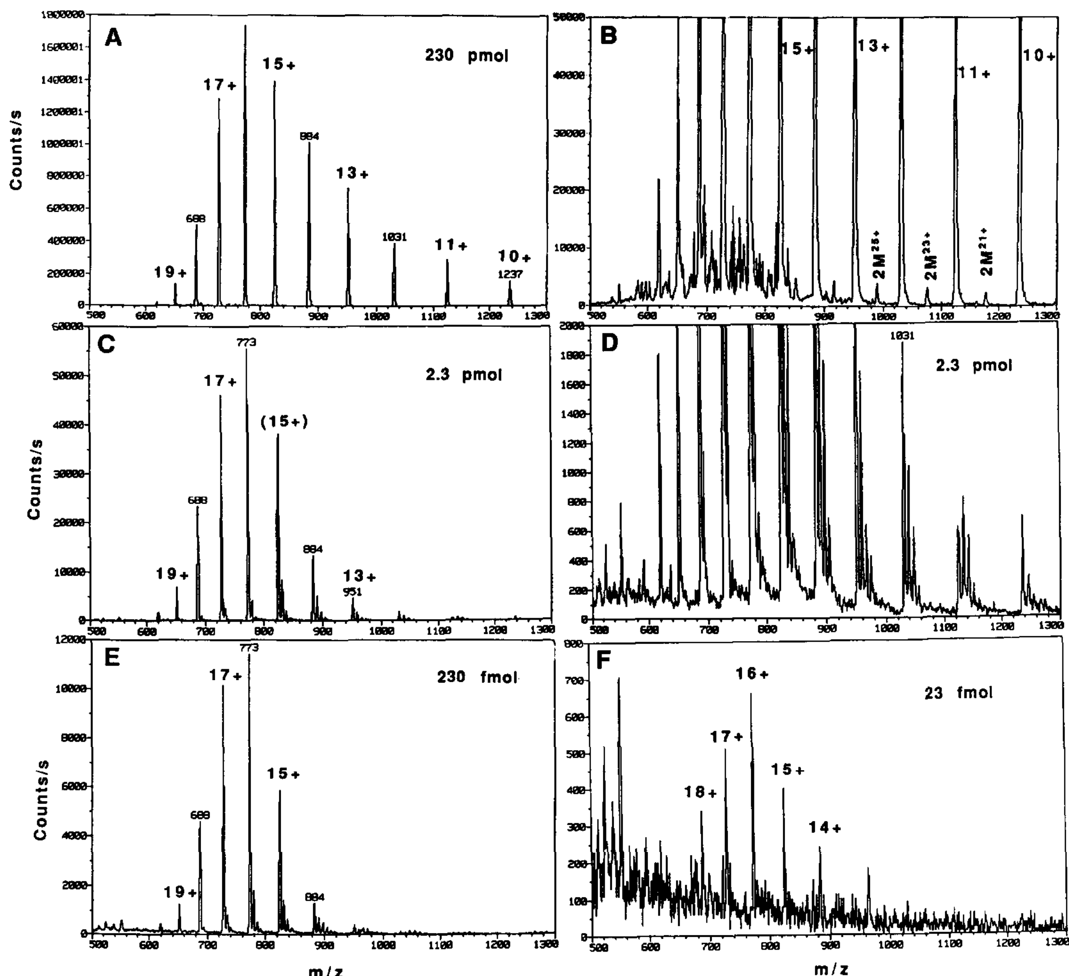

Fig. 1. ESI-MS spectra of cytochrome *c* obtained at sample flow-rates of 1 μl/min for sample concentrations ranging from $1.5 \cdot 10^{-4}$ *M* to $1.5 \cdot 10^{-8}$ *M*, consuming from 230 pmol (A) to 23 fmol (F) during 90-s spectrum acquisition periods.

a nearly linear correlation between peak intensity and concentration is still obtained for the lower charge states.

One of the most striking aspects of the ESI process is that it appears to approach unit ionization efficiency. Even at *present levels* of instrument performance, it is conceivable that useful spectra of small proteins such as cytochrome *c* might be obtained with as little as 1 fmol. The "background" in Fig. 1F is likely due to trace impurities (in solvents, on capillary surfaces, etc.). The actual detector "noise" amounts to <4 counts/s. Thus, a more limited scan range (*e.g.*, *m/z* 600–1000) and reduced background might accomplish this goal. However, such a situation would be realistic only for very clean samples (unlikely for "real world" applications) or with on-line sample clean-up and separation using capillary electrophoresis. Since currently

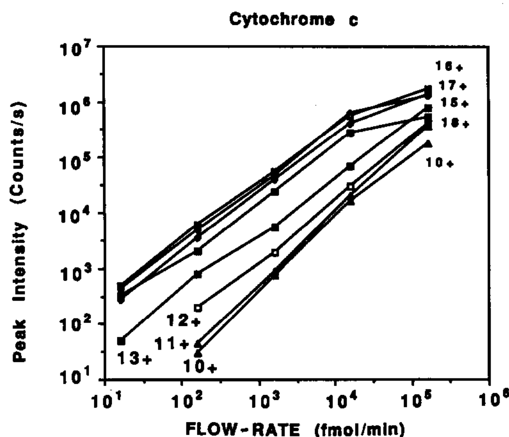

Fig. 2. Intensities of various charge states for ESI-MS of cytochrome *c* as a function of concentration (see Fig. 1).

only *ca.* 10^{-5} of all ions formed by ESI are typically detected, attention to the factors limiting instrument performance may provide a basis for extension of ESI-MS for proteins into the attomole regime.

The ability to obtain spectra for higher-M_r compounds is also crucially dependent upon instrument performance, but at very high M_r is ultimately limited due to the charge state distribution. As M_r increases, so does the average number of charges, the number of charge states, and the "peak width" as evident from our previous publications[4,31]. The increased peak width is ascribed to currently unresolved contributions of solvent attachment, charge carrying adducts (either anionic or cationic), and, perhaps most importantly, sample heterogeneity[4]. As shown in Table II these factors reduce the maximum peak intensity obtainable. For example, we expect that a protein of M_r 10 000 would give a maximum peak intensity about a factor of 50 lower than for a singly charged species *at concentrations which saturate the ESI process.* Saturation of the ESI process will occur at *ca.* 10 times lower concentration for the larger protein due to its higher charge state; thus larger sample concentrations do not increase maximum peak intensity. Detection of a protein of M_r 100 000 would require instrumental performance *ca.* 10^4 better than necessary for singly charged species ionized with equal efficiency. Our experience to date with a wide range of proteins extending to over 200 000 M_r is consistent with these expectations[31]. The variable levels of success for analysis of larger proteins obtained at different laboratories may partially reflect this aspect of instrument performance.

Useful ESI-MS for very large proteins will require both more efficient mass spectrometers as well as a reduction in the "peak width" contribution. Studies in our laboratory[31] with proteins of $M_r > 200\ 000$ have produced spectra with an unresolved "hump", consistent with expectations from Table II. Such a result is useless for M_r determination. Additionally, spectra of oligonucleotides suggest the maximum M_r addressable by ESI with current instrumentation may be somewhat lower (perhaps $< 100\ 000$) due to the slightly lower ion currents in the negative ion mode and the greater peak widths due to unresolved sodium attachment[31].

TABLE II

DEPENDENCE OF SIGNAL INTENSITY UPON MOLECULAR MASS

M_r	*Average number of charges*[a]	*Approximate number of charge states*[b]	*Peak "width" (m/z)*[c]	*Maximum intensity (ions/s)*[d]
1000	1	1	1	10^{12}
10 000	10	5	1	$2 \cdot 10^{10}$
40 000	40	20	3	$4 \cdot 10^{8}$
100 000	100	50	6	$3 \cdot 10^{7}$
200 000	200	100	(6)[e]	$(8 \cdot 10^{6})$[f]

[a] We assume the average number of charges increases linearly with M_r, and the distribution is centered about m/z 1000.

[b] Estimated from existing data and assuming all charge states are equally intense. This approximation tends to somewhat underestimate maximum peak intensity.

[c] Peak width due to sample micro heterogeneity (apparently typical of large biopolymers) and uncertain contributions of impurities, solvent adduction, etc. Approximated from available data.

[d] ESI production before sampling losses and assuming *ca.* 80% ionization efficiency. Detected ion intensities for current MS instrumentation are at least 10^4 to 10^5 lower due to losses due to inefficiencies arising from ion sampling, transmission and detection (see Table I).

[e] Peak width of 6 m/z units is too large for individual charge states to be resolved; a peak width of ≤ 4 would be required.

[f] For a peak width of 6 m/z units (see note *e*).

Finally, the sample concentration necessary to "saturate" the ESI peak intensity decreases nearly linearly with the average charge state, from *ca.* 10^{-4} *M* for a singly charged species to 10^{-6} *M* for a protein of M_r 100 000 with an average of *ca.* 100 charges/molecule. Thus, an efficiently ionized peptide or protein will saturate the ESI signal at sample concentrations of *ca.* 0.1 μg/μl for a 1 μl/min flow-rate, if ions are produced efficiently with an average m/z of *ca.* 1000. A proportionally lower ionization efficiency and threshold for ESI saturation are obtained at higher flow-rates since ESI ion current is nearly independent of flow-rate.

CONCLUSIONS

Within the past year, ESI-MS has exploded onto the biochemical community, with new applications developing at an increasing pace, especially for peptide and protein analysis. Currently, well over 100 large polypeptides and proteins (a conservative estimate), with at least half of these materials having $M_r > 10\,000$, have been successfully analyzed[31].

The correspondence between CE and ESI flow-rates and the fact that both are facilitated by (and primarily used for) ionic species in solution provide the basis for an extremely attractive combination[6,8] based upon ESI-MS. Small peptides are easily amenable to capillary zone electrophoresis (CZE)–MS analysis with good reproducibility. High efficiency separations of a series of dynorphin and enkephalin peptides have been demonstrated, with over 250 000 theoretical plates obtained by CZE–ion spray-MS[5]. Complex mixtures of peptides generated from tryptic digestion of large proteins are well suited to CZE–MS analysis, as shown by Lee *et al.*[7]. Since trypsin

specifically cleaves peptide bonds C-terminally at lysine and arginine, the resulting peptides will tend to form doubly charged as well as singly charged molecular ions. This allows most large tryptic peptides to be within the m/z range of most quadrupole mass spectrometers. Lee *et al.*[7] have demonstrated this approach for a tryptic digest from recombinant bovine somatotropin. Doubly charged molecular ions of the peptides dominate the mass spectrum. A CZE–MS daughter ion spectrum from one of the components was used to confirm the identity of a hexapeptide. Initial application of CZE–MS to proteins has been demonstrated at our laboratory[32].

Capillary isotachophoresis (CITP) is an attractive complement to CZE, and is ideally suited for combination with mass spectrometry. We have demonstrated the feasibility of CITP–MS for quaternary phosphonium and ammonium salts, amino acids, and catacholamides[6] and various polypeptides[8]. CITP is well suited to low concentration samples where the amount of solution is relatively large, whereas CZE is ideal for the analysis of minute quantities of solution. Sample size which can be addressed by CITP are much greater (>100-fold) than CZE. CITP results in concentration of analyte bands, which is in contrast to the inherent dilution with CZE. Electromigration injection allows effective sample volumes to be much larger still due to enrichment during migration into the capillary from low-ionic-strength samples[6]. Detection limits of approximately 10^{-11} M have been demonstrated for quaternary phosphonium salts, and substantial improvements appear feasible[6]. Analytes elute in CITP as bands where the length of the analyte band provides information regarding analyte concentration. Most importantly, however, is that CITP provides a relatively pure analyte band to the ESI source, without the large concentration of a supporting electrolyte demanded by CZE. Thus, CITP–MS has the potential of allowing much greater sensitivities (and analyte ion currents) than feasible with CZE–MS due to more efficient analyte ionization. The relatively wide and concentrated separated bands in CITP facilitates MS–MS experiments (which often requires more concentrated samples than tolerated by CZE). These characteristics make CITP–MS–MS well suited for characterization of enzymatic digests of proteins[9]. Recent results also suggest that useful sequence-related information may be obtained by direct MS–MS of proteins[33].

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