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On-line microdialysis of proteins with high-salt buffers for direct coupling of electrospray ionization mass spectrometry and liquid chromatography

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Abstract

Mass spectrometry (MS) is one of the most powerful instrumental techniques for protein analysis. The electrospray ionization (ESI) approach is known to be very gentle and at the same time compatible with liquid separation techniques such as HPLC and CE. However, ESI is known to be susceptible to salts and impurities, which often cause a dramatic decrease in sensitivity due to the suppression of the ionization of the product of interest. For this reason, LC–ESI-MS coupling has so far been largely limited to reversed-phase chromatography with its hydro–organic mobile phases. Other chromatographic techniques are typically ''linked'' to ESI-MS by time consuming, off-line desalting steps. On-line microdialysis has been proposed as a solution to this dilemma. In this paper, we introduce an improved microdialysis system, which enlarges the number of putative applications, thus allowing chromatographic separations of biological compounds to be directly coupled to MS detection with little to no loss in time or chromatographic resolution. Examples include separations by affinity, ion-exchange and size-exclusion chromatography, all of which were connected successfully to the ESI-MS detector via the on-line microdialyzer. We propose that, using this system, any kind of chromatography technique can be coupled to ESI-MS, thus enabling for example application in quality control or process monitoring of many bioproduction and downstream processes.

Keywords: Electrospray ionization; Interfaces, LC–MS; Microdialysis; Proteins

1. Introduction

Mass spectrometry (MS) represents a powerful technique for protein characterization. In the chromatography community, the electrospray ionization approach (ESI-MS) has received much attention, since it can in principle be easily coupled to high-resolution separation techniques such as HPLC. However, to date ESI-MS is mainly coupled to reversed-phase chromatography due to the fact that this ionization technique is highly susceptible to the presence of salts and other small molecular impurities in the sample. Techniques such as ion-exchange, hydrophobic interaction and affinity chromatography, which typically employ high salt buffers at some point of the separation, are a priori less well suited to such a coupling. These chromatographic techniques, on the other hand, are very popular in protein separation since they tend to preserve the biological activity and the native structure of the proteins.

The possibility of connecting ESI-MS to such ''high salt'' chromatographic techniques has been

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investigated in the past [1–4]. Typically, however, off-line steps are used [5] for the removal of the contaminants. Such off-line sample manipulation is often time-consuming and increases the chance of sample contamination and/or sample degradation. The ability of coupling ESI-MS directly to various chromatographic procedures used in protein purification would certainly offer an invaluable tool for accelerating the pace at which protein mixtures could be analyzed and identified. This would be most valuable for the on-line quality control and process monitoring of many production and purification processes in molecular biotechnology. Furthermore, as ESI-MS requires only soft ionization of the proteins, one can even envisage a real-time analysis of protein–protein interaction using this approach. However, with the exception of reversed-phase chromatography, chromatographic separations are at present not routinely monitored by direct ESI-MS due to the difficulties involved in coupling ESI-MS to chromatographic techniques using high-salt buffers. Even if a signal was obtained in isolated cases, e.g. by using only low salt buffers, no accurate identification of the tested proteins was usually possible due to the large number of adduct peaks and the loss of sensitivity.

Different investigators have described systems to overcome the problem of salt contamination. The most successful method for desalting is perhaps the 2-D chromatography using a reversed-phase column. However, such a system is rather complicated to set up and use and the approach is in addition time-consuming. Other solutions based on ion capture [6,7] have been proposed but unfortunately, they cannot be used for extended periods of time due to the rapid saturation of the ion-capture module. Perhaps most promising is at present the work of Liu and collaborators [8–10], who proposed the use of on-line-dialysis for biological samples such as nucleic acids and small proteins. If the results obtained by Liu and Verma with ion-exchange chromatography of small proteins [9] are extrapolated, on-line dialysis should indeed enable the high-throughput MS analysis of proteins eluting from liquid chromatography columns in the presence of salt ions.

In this paper, a modular approach to such an on-line dialysis coupling is described, which allows, for example the rapid exchange of any part of the on-line dialyzer. Moreover, our on-line dialyzer can regulate the temperature of the buffer passing through it with an integrated temperature regulator; a certain advantage, since it has been previously demonstrated [9] that precise temperature control is necessary in order to achieve complete desalting of the sample. In addition to ion-exchange chromatography several other chromatographic techniques using salt-containing buffers were coupled to an ESI-MS using this interface. Moreover, the use of ESI-MS detection was extended to the on-line-characterization of fairly large proteins, most notably antibodies with a molecular mass of 150 000 g/mol).

2. Experimental

2.1. Reagents

All the proteins (with the exception of the recombinant antibody, see below), as well as the chemicals for buffer preparation were purchased from Sigma (Buchs, Switzerland). Ultra-pure water, produced by a Millipore purification system, was used for buffer preparation. The antibody used in the demonstrations is produced as recombinant protein in CHO cells and in serum-free medium in our center [11].

2.2. Instrumentation

An Agilent 1100 HPLC system from Agilent Technologies (Waldbronn, Germany) was used for all experiments. The system consists of a high-pressure binary pump, a degasser and a UV detector (detection wavelength set at 280 nm). Samples were injected with a 7725 Rheodyne valve (Cotati, CA, USA) for anion-exchange and size-exclusion chromatography experiments and with a P1 model peristaltic pump for the Protein A affinity chromatography experiments. All tubing and fittings were from Upchurch Scientific (Oak Harbor, WA, USA). The mass spectra were acquired on a LCT mass spectrometer from Micromass (Manchester, UK). This instrument combines an electrospray ionization source with a TOF (time-of-flight) mass analyzer.

2.3. Chromatographic methods

Protein A affinity chromatography was carried out on a C10/10 column from Pharmacia (Uppsala, Sweden) packed with rProtein A Sepharose Fast Flow (Pharmacia). The column was equilibrated with 50 m*M* sodium phosphate buffer (pH 7.0) containing in addition 150 m*M* sodium chloride. The samples were prepared in the same buffer. Elution was performed with a 100 m*M* glycine buffer (pH 3.0) at a flow-rate of 0.5 ml/min.

Anion-exchange chromatography was performed on a 30 cm×4.6 mm Amicon PAE-1000A column from Millipore (Bedford, MA, USA). Buffer A was a 10 m*M* Tris–HCl buffer (pH 8.7) and buffer B a 10 m*M* Tris–HCl buffer (pH 8.7) containing in addition 1 *M* sodium chloride. The proteins were eluted with a linear gradient from 0% B to 40% B in 30 min and at a flow-rate of 1.3 ml/min. The sample consisted of a mixture of 10 mg/ml horse myoglobin (HM) and 10 mg/ml bovine hemoglobin (BH) in buffer A at a ratio of 3:1.

Size-exclusion chromatography was performed on a 30 cm×4.6 mm TSKgel Super SW3000 column from TosoHaas (Montgomeryville, PA, USA). The mobile phase was a 5 m*M* sodium phosphate buffer (pH 7.0) containing in addition 10 m*M* sodium chloride. The flow-rate was 0.2 ml/min and the sample volume 5 μl. Soybean trypsin inhibitor (STI) at a concentration of 10 mg/ml and HM at a concentration of 20 mg/ml (ratio 1:2) were used as sample.

2.4. Microdialysis module

The typical LC–MS configuration used in the experiments involving the on-line microdialyzer is shown in Fig. 1. The active part of the microdialyzer is a regenerated cellulose hollow fiber (15 cm×200 μm I.D.×216 μm O.D.) from Spectrum (Houston, TX, USA) with a molecular mass cut-off (MWCO) of 13 000. Both extremities of the fiber were glued to micro-tight tubing sleeves (330 μm I.D.×635 μm O.D.). PTFE tubing (500 μm I.D.×2 mm O.D.) was used to connect the hollow fiber to the fused-silica capillary (100 μm I.D.). The dialysis fiber was inserted in a cassette constituted of two plates (20 cm×4 cm×3 cm) screwed together to form a 1.8 mm deep channel. Two polyether ether ketone (PEEK) tubings (1/16 in. O.D.×0.030 in. I.D.; 1 in.=2.54 cm), inserted though the upper part of the cassette, allowed the circulation of the dialysis buffer in the channel. The lower part was closed with a third plate containing the heat exchanger system used for thermostating the device (Fig. 1 insert). The dialysis channel was filled with acetic acid at a concentration of 5 m*M*, preheated to 50°C and pumped with a Masterflex C/L pump from Cole Parmer (Vernon Hill, IL, USA) at a flow-rate of 350 μl/min. The inlet fused-silica capillary was connected to the LC system by a micro-splitter valve (Upchurch) placed between the column and the detector. The outlet capillary was attached directly to the probe of the mass spectrometer. The LC eluent flow was split at a flow-rate of 10 μl/min into the microdialyzer. To enhance protein ionization, a 1:1 (v/v) mixture of (a) 10% formic acid in water and (b) 2% formic acid in water–acetonitrile (1:1) was used as sheath liquid.

2.5. ESI-MS conditions

The LCT mass spectrometer was used in positive ionization mode for all experiments. The ES+ source parameters were adjusted as a function of the molecular mass (M_r) of the proteins tested. For the antibody analysis, the parameters were: capillary voltage 3.2 kV, sample cone voltage 55 V, extraction cone voltage 1 V, radio frequency (RF) lens voltage 400 V, desolvation temperature 150°C, source temperature 110°C, and desolvation flow-rate 500 l/h. For the other proteins the parameters were as follows: capillary voltage 3.5 kV, sample cone voltage 45 V, extraction cone voltage 3 V, RF lens voltage 300 V, desolvation temperature 200°C, source temperature 120°C, and desolvation flow-rate 500 l/h.

3. Results and discussion

The possibility of using hollow fibers for on-line dialysis of biological samples has been demonstrated previously both for nucleic acids [8] and for small proteins [9,10]. The possibility of using such a device for coupling various LC techniques to ESI-MS has been discussed in this context. However, for

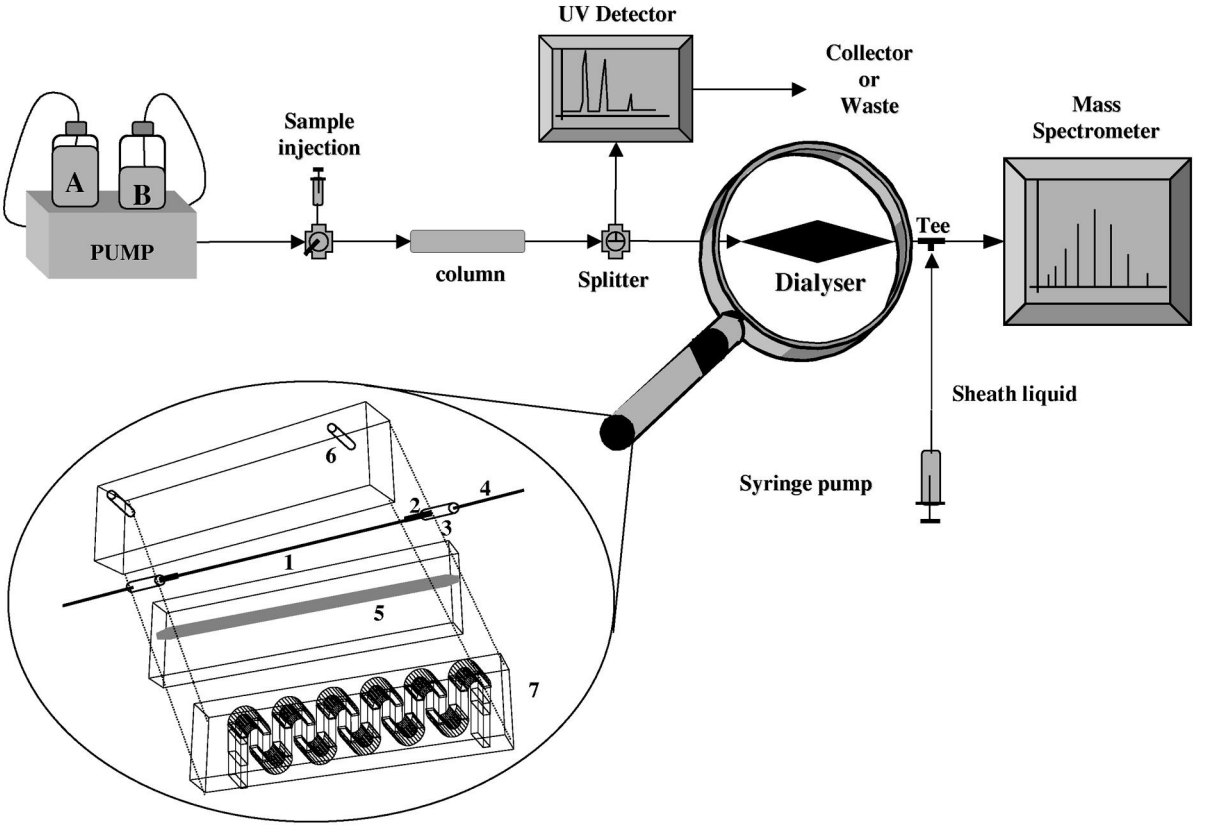

Fig. 1. Schematic presentation of the direct coupling of liquid chromatography and electrospray ionization mass spectrometry through an on-line microdialyzer. Insert: microdialyzer with (1) hollow fiber, (2) micro-tight tubing sleeves, (3) PTFE tubing, (4) fused-silica capillary, (5) dialysis channel, (6) dialysis buffer inlet, and (7) lower part of the module including heating system.

many chromatography techniques commonly used in protein purification and in protein characterization so far, no data have been shown demonstrating the possibility of coupling them to ESI-MS using such a device.

Our development and optimization of such an approach started with a series of experiments during which a control protein (horse heart myoglobin) diluted in different buffers was injected into the ESI-MS system by infusion injection via the microdialyzer (data not shown). These preliminary experiments were used to determine the boundary conditions compatible with protein analysis by ESI-MS. The necessity for strict temperature control in the microdialyzer also became obvious during these experiments. Using these parameters and conditions, we subsequently investigated the possibility of on-line coupling various LC separation methods to ESI-MS by analyzing proteins with different molar masses (Mw) as well as different isoelectric points (p*I*).

3.1. Coupling of Protein A affinity chromatography with ESI-MS

Protein A affinity chromatography based on rProtein A ligands was used as a pertinent example to test the possibility of using ESI-MS detection in connection with affinity capturing in general. The Protein A approach is widely used in biotechnology for the purification of antibody molecules, which are amongst the most frequently used agents in biology and medicine. Antibody molecules (more specifically IgGs) have high relative molecular masses (~150 000

g/mol) and consist of four polypeptide chains linked together via disulfide bridges. In order to obtain a good ionization ratio for such large molecules, it is recommended to use a high percentage of formic acid solution (10%) as sheath liquid.

The elution buffer for the Protein A column was the commonly used 100 m*M* glycine (pH 3.0) buffer, which had previously been shown to be capable of quantitatively eluting the antibody used here from the column [12]. When the eluted antibody fraction was directly injected into the ESI-MS, no signal was observed and the total ion current (TIC) trace indicated that no ionization of the antibody occurred, probably due to the high concentration of glycine molecules in the elution buffer (Fig. 2A). When the microdialyzer module was used to couple the Protein A column to the ESI-MS, we were able to observe a peak in both the UV and the TIC chromatograms (Fig. 2B). These peaks correspond to the antibody molecule, demonstrating the efficiency of the on-line dialysis module for removing low-molecular mass contaminants (such as glycine) present in the elution buffer. The resulting TIC signal is somewhat broader than the UV trace due to interactions between the antibody molecule and the cellulose hollow-fiber used in the microdialyzer. Furthermore, the TIC signal appears later than the UV trace because the MS detector is placed downstream of the UV detector (increased void volume).

Recombinant antibody molecules are often produced as a heterogeneous mixture, e.g. with different glycosilation patterns. This decreases the resolution of the Protein A separation and consequently, accurate masses can often not be calculated. However, affinity capture is commonly used early on during a bioseparation process and under such circumstances the main goal may well be to identify the general nature of the product rather than to attribute a precise mass to the molecule eluting from the Protein A column. Fig. 3 compares the data obtained by directly coupled Protein A-ESI-MS with those obtained by infusion injection into the ESI-MS system under identical ionization conditions of the corresponding antibody standard obtained by a three-step off-line purification protocol [13]. Clearly both spectra show the same profile. It can therefore be deduced that on-line MS coupling can indeed rapidly generate enough information on the nature of the target product to serve as a decision tool for further downstream processing, thus bypassing classical, time-consuming protocols such as polyacrylamide gel electrophoresis and Western blotting.

3.2. Coupling of anion-exchange chromatography with ESI-MS

Ion-exchange chromatography (IEX) is another popular method of preparative protein chromatography. It is highly efficient, the corresponding columns tend to have high capacities, while the biological activity of the proteins is usually maintained to the highest possible degree. IEX is often used directly after the affinity capturing step. The method generally requires the use of a gradient of increasing salt concentration for protein elution, thereby drastically decreasing the likelihood of successfully employing directly ESI-MS for the analysis of the eluted substances. To evaluate the usefulness of our interface in this context, we coupled an anion-exchange column to the mass spectrometer via the microdialyzer module.

A mixture of two proteins with close isoelectric points, namely myoglobin from horse heart and bovine hemoglobin (pI=7.36 and 8.19, respectively) was used in the experiments. Although IEX is a standard method in biotechnology for protein purification, it is sometimes difficult to predict the elution behavior in the case of mixtures of proteins with very close pI values. To identify the different products in IEX beyond any doubt, the separation conditions and the detection systems have to be fine-tuned for the precise identification of all the compounds present in the solution. In the case considered here, the obtained chromatogram (Fig. 4A) showed some peculiarities, e.g. with regard to the shape of the second peak, which is rather broad.

Two hypotheses were formulated. It is possible that the first peak is a mixture of both proteins (not resolved due to the similar pI values) and the second peak represents simply a contaminant. However, it could also be that a separation was achieved as planned, in which case the first peak corresponds to pure myoglobin and the second peak to the hemoglobin. In the latter case the broadness of the second peak could be caused by secondary interactions of hemoglobin with the anion-exchange matrix. The

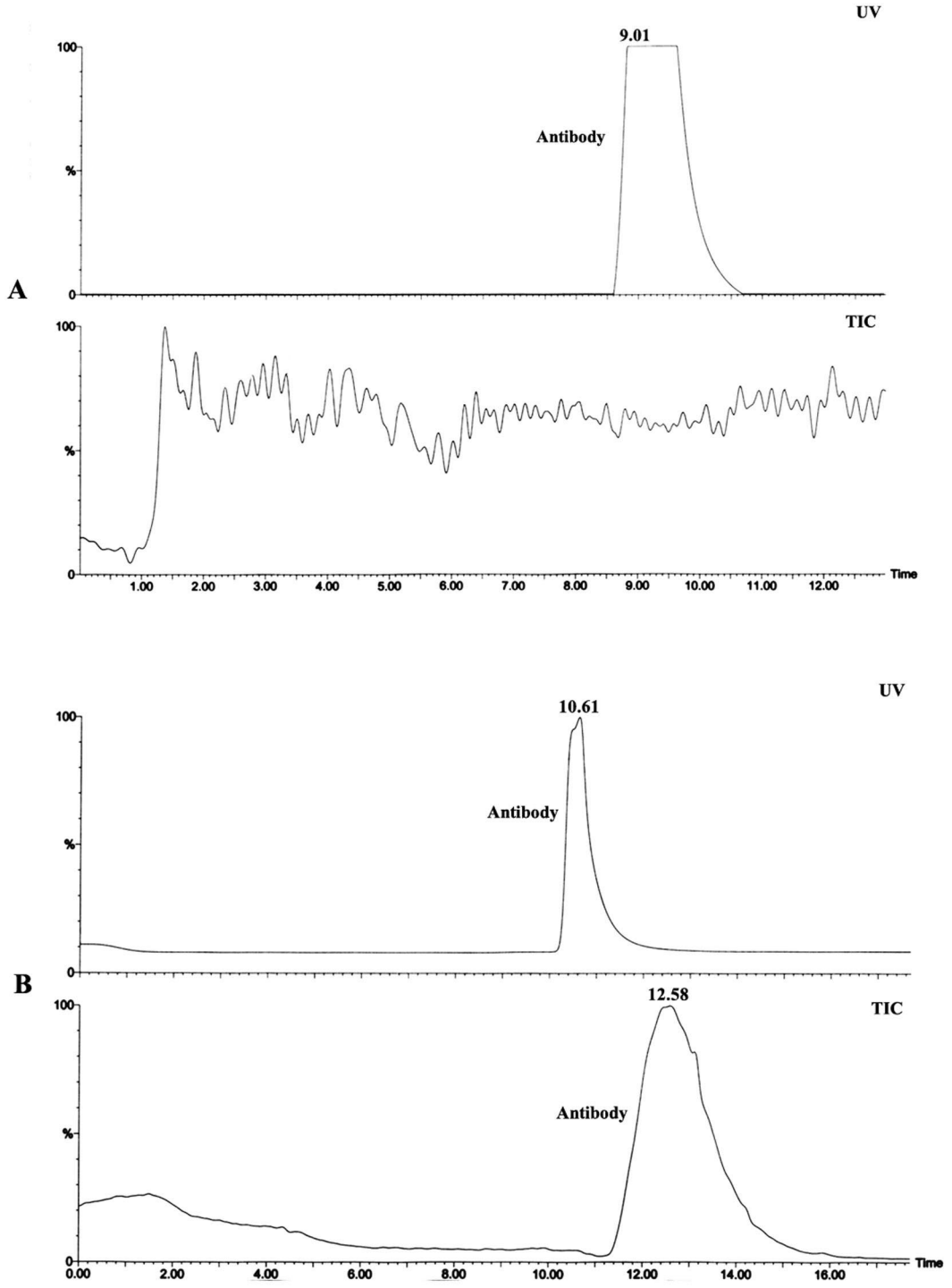

Fig. 2. Coupling of Protein A affinity chromatography and ESI-MS for analysis of a recombinant antibody. (A) Coupling without on-line microdialyzer system, (B) coupling with on-line microdialyzer system in place. For both A and B, top trace: UV chromatogram at 280 nm, bottom trace: TIC (total ion current) chromatogram. Time scales in min.

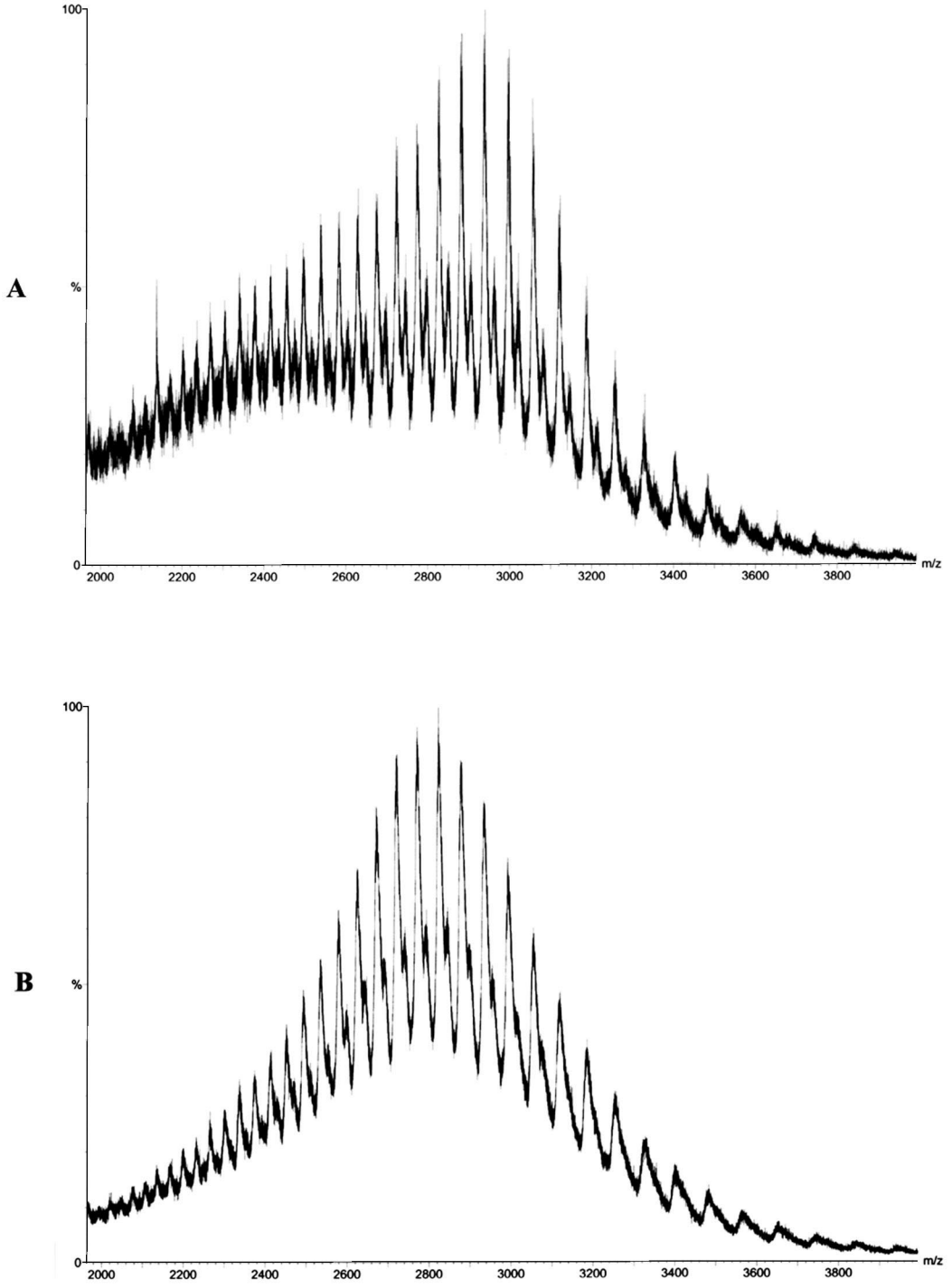

Fig. 3. ESI-MS spectra of the recombinant antibody. (A) Spectrum obtained from Protein A-on-line microdialyzer–mass spectrometer coupling, (B) spectrum of the corresponding antibody standard obtained after a three-step purification process and infusion injection through the microdialyzer.

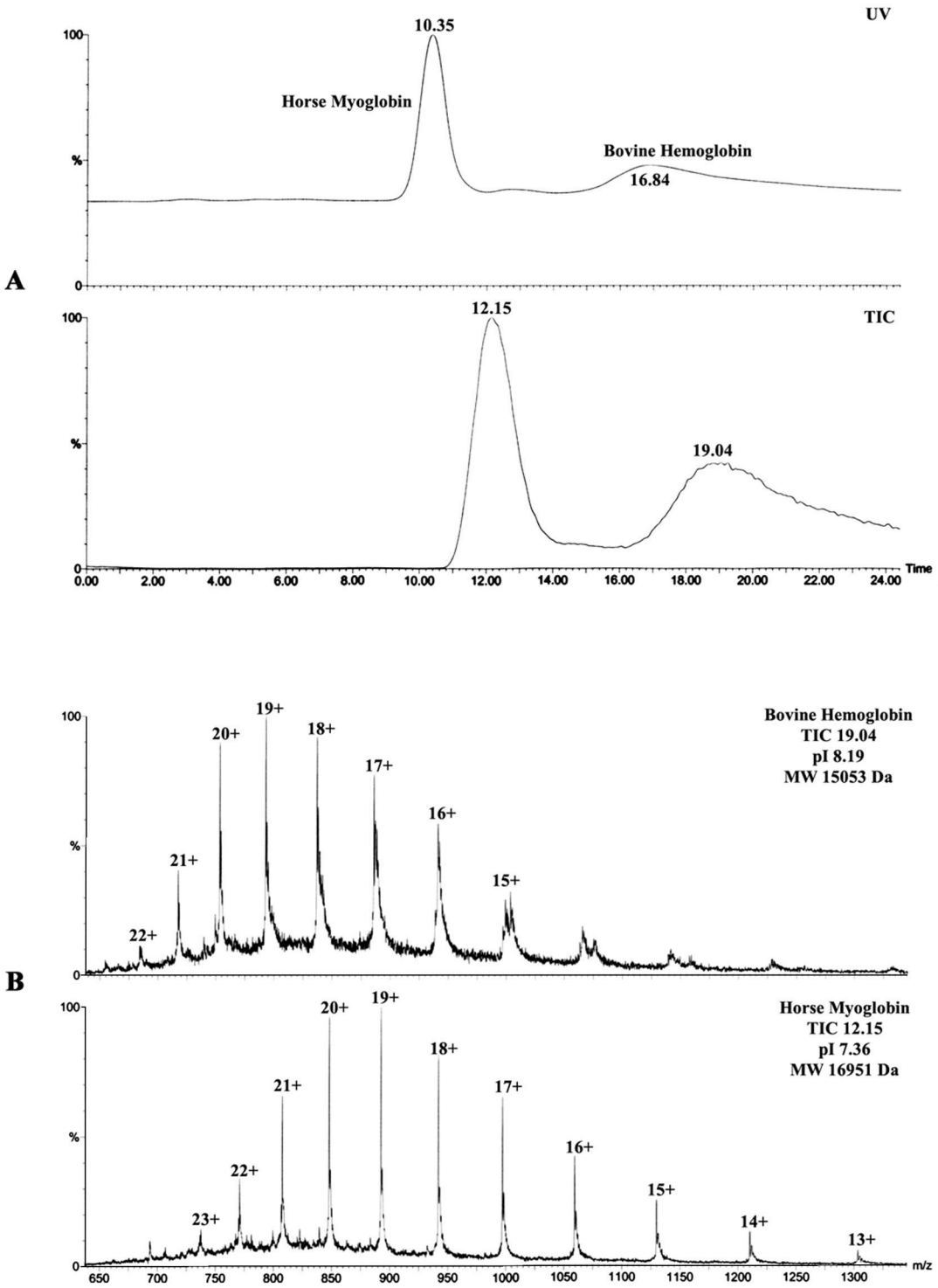

Fig. 4. Coupling of anion-exchange chromatography and ESI-MS. (A) Top trace: UV chromatogram at 280 nm, bottom trace: TIC chromatogram. (B) Mass spectra of the two proteins, top trace: bovine hemoglobin spectrum from TIC peak at 19.04 min, bottom trace: horse heart myoglobin spectrum from TIC peak at 12.15 min. Time scales in min.

on-line analysis of the eluate by on-line ESI-MS (Fig. 4B) clearly demonstrated that the second hypothesis was the correct one and that the first peak in the chromatogram corresponded to myoglobin and the second to hemoglobin (see for example the calculated molecular masses). No contaminants were observed for either protein. Coupling IEX to MS via a microdialyzer thus permits the analysis in real-time of the protein present in a substance zone and also the precise identification of their relative molecular masses.

3.3. Coupling of size-exclusion chromatography and ESI-MS

Size-exclusion chromatography (or gel filtration) is used as the last step in the downstream procedures of many recombinant proteins (''polishing step''). The corresponding chromatograms are also used to demonstrate the purity of the product, required in the case of, for example, medical applications. In order to demonstrate the potential of using ESI-MS analysis directly coupled to size-exclusion chromatography (SEC), a mixture of two proteins, namely myoglobin from horse heart and soybean trypsin inhibitor with fairly similar molar masses (17 000 and 20 000 g/mol, respectively) was separated on an SEC column. The protein mixture was well resolved by the SEC column, as demonstrated by the corresponding UV chromatogram (Fig. 5A, top) but the corresponding TIC trace showed, by comparison, uncommonly broad peaks (Fig. 5A, bottom).

Some peak broadening occurred in all the applications discussed so far. It is due to the (small) void volume in the microdialyzer chamber that the sample crosses on its way to the MS system. This effect is amplified by the relatively low flow-rate used in gel filtration. However, due to the fact that TOF analysis can be carried out at high scanning speeds, the resolution decrease due to the peak broadening is more than compensated by the ability to generate chromatograms that can be easily ''interpreted'' by MS. Due to the high content of multiple charged ions for each proteins in the sample, it was possible to obtain a precise analysis of the spectra (attribution of the relative molar mass as well as the purity). The spectrum of the soybean trypsin inhibitor shows a pattern indicating the presence of both isoforms of the protein (Fig. 5B, bottom), whereas the spectrum of the myoglobin (Fig. 5B, top) showed that the contaminant originally present in the Sigma product (peak at 17 min) had been quantitatively removed by the SEC step.

This experiment clearly demonstrated the orthogonality of SEC and MS. While the UV detection yields information on the purity of the samples during the separation process, the MS measurement allows calculation of the precise mass of the compounds (identification) and confirms the purity of the products.

4. Conclusion

In the present work, we describe an important step towards the direct coupling of ESI-MS detection with LC methods requiring high salt buffers. The proposed dialyzer module and the conditions for using it allow efficient on-line desalting of the LC eluates. Furthermore, no sodium adducts of the analytes are formed after injection into the MS, which makes the treatment of the spectra easier and faster. As a result, the coupling of ESI-MS is no longer limited to reversed-phase chromatography but can be achieved successfully with all types of liquid chromatography. The results obtained with the on-line microdialyzer coupling of LC and MS can be used as a rapid decision-making tool during downstream processing in biotechnology for the production and the characterization of recombinant proteins. This will lead to a gain in time and help to avoid eventual mistakes due to excessive sample handling.

There is clearly some room for improvement. For instance, the use of hollow fibers with higher buffer exchange properties could serve to shorten the microdialyzer module and therefore reduce the void volume to avoid peak broadening. A more inert material than the regenerated cellulose could decrease (the small residual) non-specific adsorption. In the end we hope that one day this gentle automated method could complement two-dimensional gel electrophoresis–spot identification by matrix-assisted laser desorption ionization (MALDI) MS approaches used in the evolving field of proteomics, notably for analyzing protein–protein interactions.

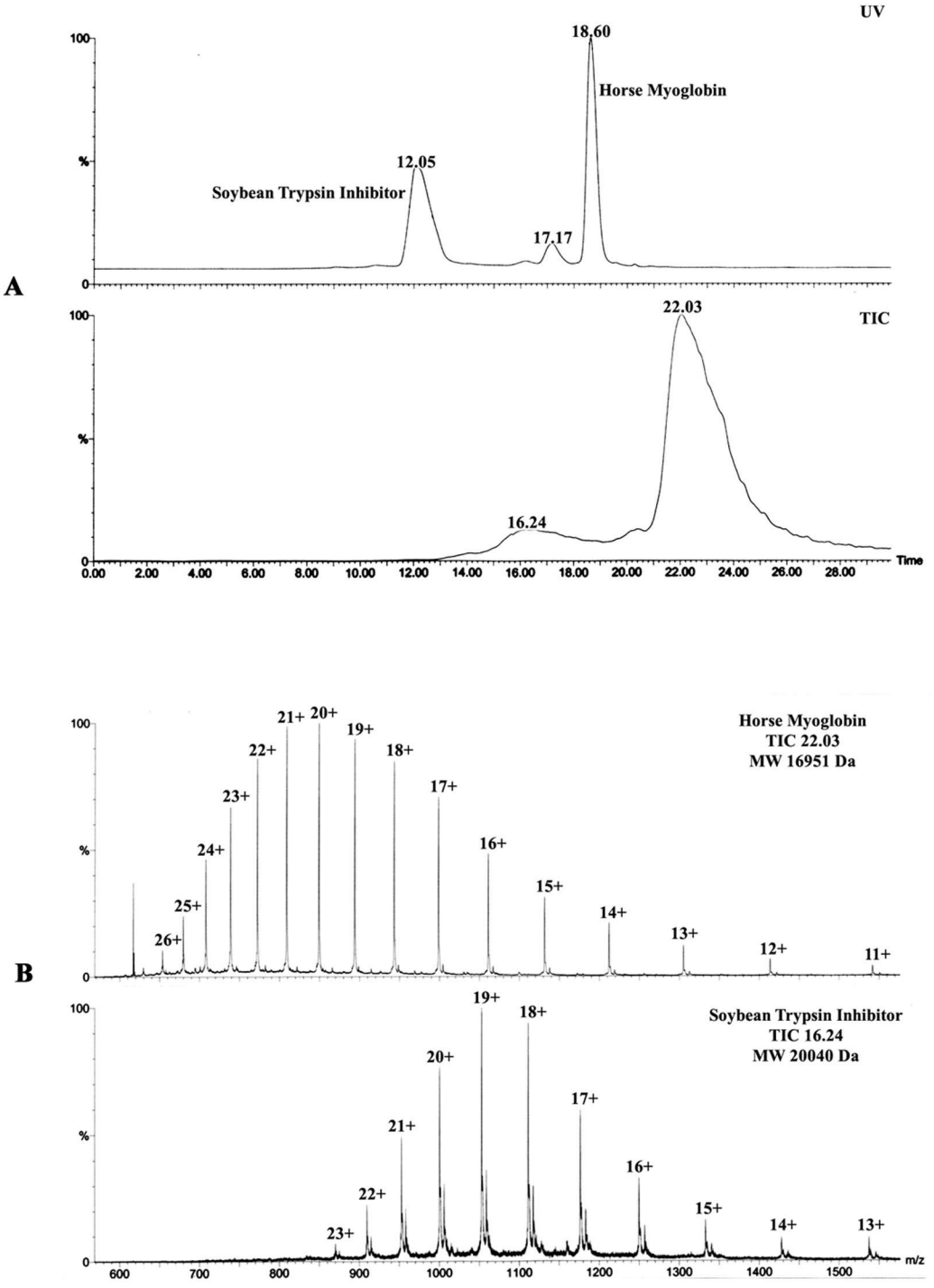

Fig. 5. Coupling of size-exclusion chromatography and ESI-MS. (A) Top trace: UV chromatogram at 280 nm, bottom trace: TIC chromatogram. (B) Mass spectra of the two proteins, top trace: horse heart myoglobin spectrum from TIC peak at 18.60 min, bottom trace: soybean trypsin inhibitor spectrum from TIC peak at 12.15 min. Time scale in min.

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