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Short Communication

HOLLOW-FIBER FLOW FIELD-FLOW FRACTIONATION: A GENTLE SEPARATION METHOD FOR MASS SPECTROMETRY OF NATIVE PROTEINS

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Low-impact ionization sources like electrospray ionization (ESI) and matrix-assisted, laser desorption/ionization (MALDI) equipped with time-of-flight (TOF) mass analyzers provide intact protein analysis over a very wide molar mass range. ESI/TOFMS provides also indications on the higher-order structure of intact proteins and non-covalent protein complexes.[1,2] However, direct analysis of intact proteins mixtures in real samples shows limited success, mainly because spectra become very complex to interpret. This is also due to sample contaminants, and to the mechanism of competitive ionization in ESI or MALDI. Rapid and efficient sample clean-up and separation methods can significantly enhance the power of TOFMS for intact protein analysis. However, if protein native conditions want to be maintained, the methods should affect neither the three-dimensional structure nor the non-covalent chemistry of the proteins.

Reversed-phase (RP) HPLC, size-exclusion chromatography (SEC), and capillary zone electrophoresis (CZE) are on-line or off-line coupled to ESI/TOFMS or MALDI/TOFMS. In fact, these separation methods often show limitations when applied to the analysis of native proteins. Organic modifiers and saline buffers are required in the case of RP HPLC or CZE. They can induce protein degradation or affect ionization when MS is performed after separation. High voltages used in CZE can contribute to alter proteins from their native form. In the case of high molar mass proteins, SEC is scarcely selective, and barely able to detect protein aggregates. Sample entanglement/adsorption on the stationary phase can also occur.

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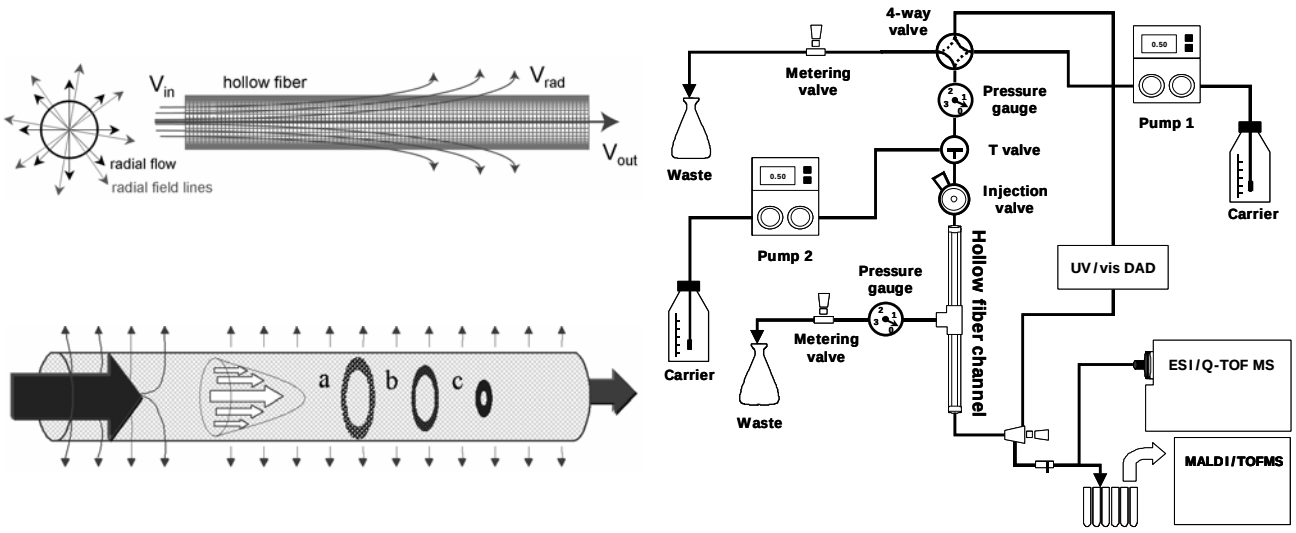
A prototype technique has been developed for gentle clean-up and separation of native proteins before TOFMS. The technique is based on flow field-flow fractionation (FlFFF), which can be applied to a broad variety of bioanalytes.[3] FlFFF recently shows very effective for non-degrading separation and further characterization of high molar mass proteins and protein oligomers.[4] This technique employs a piece (20-25 cm in length) of submillimiter-sized hollow-fiber membrane (HF) for microdialysis, which is used as microcolumn channel for FlFFF.[5-8] HF microdialysis had been successfully coupled with MS to separate protein samples into two fractions, one being the permeate containing low molecular weight compounds, and the other being the retentate containing the proteins.[9,10] When a HF membrane is used for FlFFF, also the retentate components (proteins) are separated, which is an advantage for their further MS analysis.

In HF FlFFF a mobile phase fluid enters the HF inner side and, while it flows along the HF, it is divided into two parts. Part of the flow radially exits through the HF pores, while the rest longitudinally flows all along the HF length, and it finally exits from the HF outlet. The HF FlFFF separation principle is represented in Figure 1a. Separation of sample components is structured by the combined action of the longitudinal and the radial flow, the latter acting as the hydrodynamic field for the FlFFF mechanism. HF FlFFF shows interesting features for TOFMS analysis of intact proteins. Low channel volume (in the order of few microliters) reduces sample dilution. This maintains high MS detectability and analysis time compatible with the rapid TOFMS methods. Low flow rate conditions do not necessarily require flow splitting when the channel outlet is on-line connected to the ESI source. Possible disposable usage of the channel eliminates the risk of run-to-run sample carry-over and, then, spectra contamination. In-line sample clean-up eliminates low molar mass components like salts, the presence of which can affect ionization.



(a) (b)

FIG. 1 - (a) The HF FlFFF principle. The mobile phase flow (V_{in}) enters the HF channel, and it is divided in two parts: the radial flow (V_{rad}) exits through the HF pores from the inner to the outer wall of the HF; the second component longitudinally flows along the HF, and it exits from the channel outlet (V_{out}). Separation is then structured by the combined action of the axial and the radial flow, which makes sample components (a, b, c) eluting at different velocities. (b) The HF FlFFF-TOFMS system set-up.

The HF FlFFF channels are home-made by sheathing a piece of HF with two pieces of Teflon or glass tube. A tee-connection is positioned between the two tubes to make the radial flow outlet, and two hand-tight PEEK male fittings (e.g. from Upchurch Scientific, Oak Harbor, WA) are positioned at the HF inlet and outlet.[6-8] Polysulfone or polyacrylonitrile HF membranes with 10,000 to 30,000 Da pore cut-off (SK Chemicals, Seoul, Korea; Chemicore, Daejeon, Korea) have been employed. The HF FlFFF channel is inserted into a properly modified HPLC system. For on-line HF FlFFF-ESI/TOFMS operations, the source can be directly connected to the channel through a piece of PEEK tube (e.g. 0.005" I.D, 1/16" O.D. from Upchurch Scientific). When necessary, a low-volume, micro-splitter valve can be employed to reduce the flow rate to the ESI source. For off-line MALDI/TOFMS operations, fractions are collected at the HF FlFFF system outlet. Fraction aliquots of 1 μL are then spotted on the target plate. The system diagram is reported in Figure 1b. The HF FlFFF analysis protocol typically involves two steps: injection/focusing/ relaxation of the sample, and the elution. A channel make-up run with the sample is necessary whenever a new channel is employed. The values of longitudinal outlet (V_{out}) and radial flow rate (V_{rad}), and the different flow patterns required for the two analysis steps are set up by metering and switching valves (e.g. from Nupro, Willoughby, OH, and from Hamilton, Reno, NV). Samples can be injected through a HPLC port equipped with a fixed-volume loop. According to the chosen HF pore cut-off value, during focusing/relaxation the sample is in-channel purified from low molar mass components that are washed out through the HF pores.

HF FlFFF separates intact proteins according to differences in their diffusion coefficient. By on-line HF FlFFF-ESI/TOFMS it is shown that using 50 mM ammonium acetate in water (pH 7.0) as mobile phase, proteins can be desalted and separated without affecting their ionization, with their native (or pseudonative) structure maintained after separation[11]. In the spectra of fractionated horse heart myoglobin, human hemoglobin (Hb), and horseradish peroxidase, the molar mass values corresponding to the non-covalent complexes between the polypeptide chain and the heme group were found. This was not found in RP HPLC-ESI/TOFMS of same samples. Fig. 2 reports an example of HF FlFFF-ESI/TOFMS of Hb. Correlation between the molar mass values independently measured by HF FlFFF retention times and by MS allows to confirm protein aggregation features or to indicate possible changes in the protein quaternary structure, as it has been shown showed by HF FlFFF-ESI/TOFMS of BSA, and of Hb, respectively.[11]

HF FlFFF and MALDI/TOFMS are combined in order to identify the presence of supra-molecular structures and enzyme/protein impurity complexes in recombinant enzyme samples. Fig. 3 reports (a) the fractogram of Rasburicase, a recombinant enzyme drug (from Sanofi-Syntelabo, Milan, Italy), and (b) the MALDI/TOF mass spectrum of the fractionated sample. The spectrum shows the presence of oligomers (1×, 2×, 3×, 4×) after fractionation. Using a chemiluminescent enzymatic assay, it has been proven that sample fractions retained the enzymatic activity.

We are also working on HF FlFFF with TOFMS of real samples such as human blood whole serum, and to improve HF FlFFF performance. Narrow-bore HF membranes shows to increase separation efficiency, and to reduce limits of detection of protein mixtures.[12]

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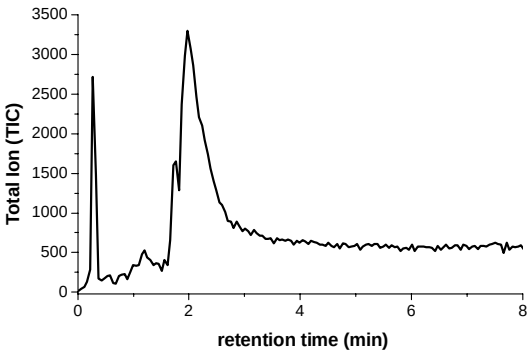
(a)



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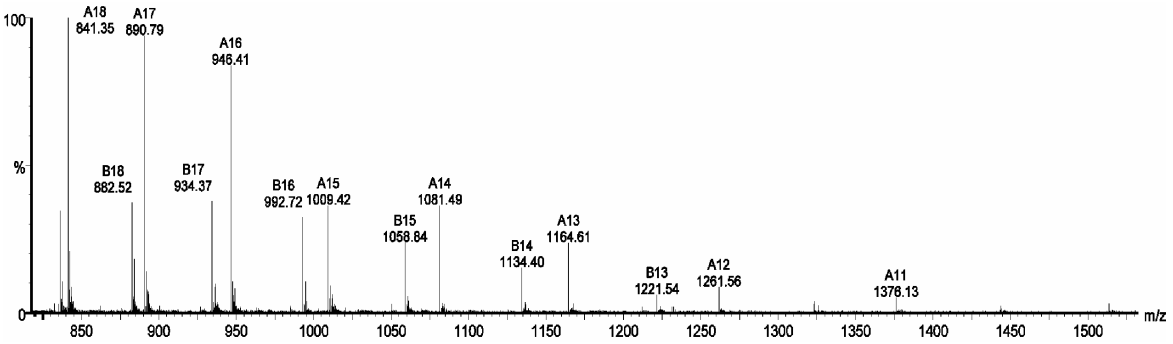
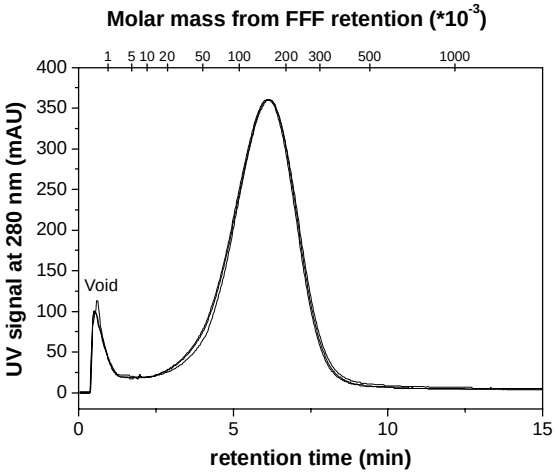
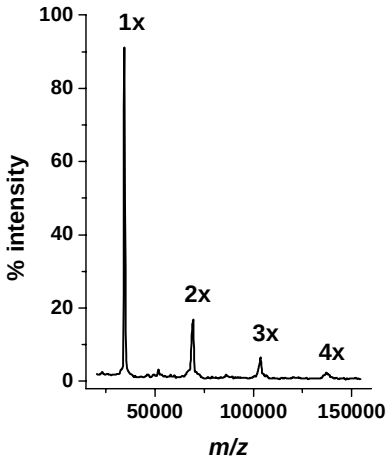


FIG. 2 - An example of HF FlFFF-ESI/TOF MS of intact proteins. Sample: human hemoglobin (Hb). Mobile phase: 50 mM NH_4CH_3COO; pH = 7.0;
V_{in} = 0.70 mL/min; V_{rad} = 0.38 mL/min (a) Total ion (TIC) fractogram.
(b) Multicharged ion spectrum.



(a)



(b)

FIG. 3 - HF FlFFF with MALDI/TOFMS of an enzyme drug. Sample: Rasburicase (Sanofi-Syntelabo, Milan, Italy). (a) HF FlFFF fractograms of three repeated runs; molar mass conversion obtained by calibration with standard proteins as reported in ref. 11. Mobile phase: 50 mM NH_4CH_3COO; pH = 7.0;
V_{in} = 0.70 mL/min; V_{rad} = 0.30 mL/min. (b) MALDI/TOF mass spectrum of the fractionated sample; Matrix: 50 mg/mL sinapinic acid in 1:1 v/v ACN/H_2O containing 0.1% v/v TFA.

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