Anal. Chem. **2005,** *77,* 47−56

On-Line Hollow-Fiber Flow Field-Flow Fractionation-Electrospray Ionization/ Time-of-Flight Mass Spectrometry of Intact Proteins

Pierluigi Reschiglian, Andrea Zattoni, Barbara Roda, and Leonardo Cinque

Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, I-40126 Bologna, Italy

Daniela Parisi and Aldo Roda*

Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, I-40126 Bologna, Italy

Fabrizio Dal Piaz†

Bioanalytical Mass Spectrometry Laboratory, Interdepartmental Center for Biotechnology Research, University of Bologna, Via Irnerio 46, I-40126 Bologna, Italy

Myeong Hee Moon‡ and Byung Ryul Min§

Department of Chemistry, and Department of Chemical Engineering, Yonsei University, Seoul 120-749, Korea

Capabilities of mass spectrometry for the analysis of intact proteins can be increased through separation methods. Flow field-flow fractionation (FlFFF) is characterized by the particularly "soft" separation mechanism, which is ideally suited to maintain the native structure of intact proteins. This work describes the original on-line coupling between hollow-fiber FlFFF (HF FlFFF), the microcolumn variant of FlFFF, and electrospray ionization/time-of-flight mass spectrometry (ESI/TOFMS) for the analysis and characterization of intact proteins. The results show that the native (or pseudonative) structure of horse heart myoglobin and horseradish peroxidase is maintained. Sample desalting is also observed for horse heart myoglobin. Correlation between the molar mass values independently measured by HF FlFFF retention and ESI/TOFMS allows us to confirm the protein aggregation features of bovine serum albumin and to indicate possible changes in the quaternary structure of human hemoglobin.

Protein regulation and interaction with surrounding species such as other proteins, protein receptors, drugs, or cell metabolites are recognized to play a fundamental role in the biological complexity of higher organisms.[1] It is acknowledged that comprehensive identification and characterization of proteins and protein derivatives in their intact form should become a fundamental task to fully understand their actual activity in biological systems. This is in fact a task of functional proteomics. Functional proteomics takes into account how native proteins interact with surrounding proteins or molecules to eventually modify the protein structure. Very accurate measurement of the actual molar mass is a prime goal to identify intact proteins and protein complexes. Mass spectrometry (MS) is the reference technique for this purpose. Electrospray ionization (ESI) is particularly suited to MS-based identification and structural characterization of intact protein and protein complexes. Because of the known ability of ESI to give multicharged ion patterns,[2] ESI-MS can be used for the analysis of intact proteins up to a mass of 200 000 Da.[3] By ESI-MS, accurate mass measures and indications on the higher-order structure of proteins and noncovalent protein complexes can be obtained.[4−7] Equipped with time-of-flight (TOF) mass analyzers, ESI/TOFMS provides characterization of intact proteins and protein complexes over a very wide molar mass range, since TOF analyzers are able to scan broad ranges of m/z values.[8−10] In the

* Corresponding author. Fax: +39(0)51300700. E-mail: aldo.roda@unibo.it.
† Current address: Department of Pharmaceutical Sciences, University of Salerno, Via Ponte Don Melillo, 84084, Fisciano (SA), Italy.
‡ Department of Chemistry, Yonsei University.
§ Department of Chemical Engineering, Yonsei University.

(1) Plavec, I.; Sirenko, O.; Privat, S.; Wang, Y.; Dajee, M.; Melrose, J.; Nakao, B.; Hytopoulos, E.; Berg, E. L.; Butcher, E. C. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 1223−1228.
(2) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64−71.
(3) Feng, R.; Konishi, Y. *Anal. Chem.* **1992**, *64*, 2090−2095.
(4) Chowdhury, S. K.; Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1990**, *112*, 9012−9013.
(5) Ganem, B.; Li, Y. T.; Henion, J. D. *J. Am. Chem. Soc.* **1991**, *113*, 7818−1819.
(6) Smith, R. D.; Loo, J. A.; Loo, R. R. O.; Busman, M.; Udseth, H. R. *Mass Spectrom. Rev.* **1991**, *10*, 339−451.
(7) Mann, M.; Hendrickson, R. C.; Pandey, A. *Annu. Rev. Biochem.* **2001**, *70*, 437−473.
(8) Fandrich, M.; Tito, M. A.; Leroux, M. R.; Rostom, A. A.; Hartl, F. U.; Dobson, C. M.; Robinson, C. V. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 14151−14155.

10.1021/ac048898o CCC: $25
Published on Web 11/19/2004

case of complex protein samples (e.g., cell lysates), direct ESI/TOFMS shows, however, limited success, mainly because the spectra are very complex to interpret. First, the spectra contain a very high number of signals due to the high number of ionized species. These species can originate not only from the different proteins present in the sample but also from the sample contaminants. The presence of sample contaminants plays an important role in terms of sensitivity and accuracy of the ESI/TOFMS methods for intact proteins. Among such contaminants, nonvolatile salts generally present in protein samples of either biological or synthetic origin give formation of adduct ions, reducing sensitivity and increasing complication in molar mass determination. This can be a particularly serious issue when proteins from bacterial cell lysates, or produced by biotechnological processes, are to be analyzed, due to the high concentration of salts present in the growing medium. Sample desalting methods are thus necessary. Second, the mechanism of competitive ionization, which is characterized by the suppression of molecular ion species of a given protein in the presence of other proteins in the mixture, also complicates spectra interpretation. Rapid and efficient separation methods able to purify the sample and affect neither the three-dimensional structure nor the noncovalent chemistry can significantly enhance the power of ESI/TOFMS methods applied to intact proteins. The increase in analytical information can be achieved in terms not only of protein identification but also of stoichiometry and characterization of the aggregation features of protein complexes.

Over almost two decades, separation techniques have been coupled with ESI-MS. First LC/ESI-MS was reported by Fenn et al.[11] in 1985, and capillary zone electrophoresis (CZE) was first coupled with ESI-MS by Smith et al. in 1987.[12] Because of the clearly superior separation efficiency of CZE over LC, CZE has rapidly gained popularity for protein characterization, and coupled with MS methods, it is considered to be among the most promising techniques for "single-cell proteomics".[13−15] However, it is recognized that some limitations could emerge if CZE-ESI-MS wanted to be applied to the analysis of intact proteins or protein complexes in native form. For instance, it is recognized that if saline buffers at relatively high ionic strength are used in CZE, they can cause problems to on-line ESI-MS, and the high voltages used in CZE can contribute to alter proteins from their native form.[16,17] When CZE uses buffers containing an organic modifier, this can cause protein dissociation into the apoprotein and the prosthetic group.[18] These difficulties are among the reasons that have up to now prevented CZE-ESI-MS from becoming a routine technique for the characterization of intact protein and protein complexes. Reversed-phase (RP) HPLC/ESI-MS is capable of analyzing and characterizing intact proteins, and even intact, noncovalent protein complexes.[19] However, when RP HPLC is used alone before ESI-MS, it seldom provides enough resolution to adequately separate proteins from complex samples. It can give sample desalting but cannot tolerate high salt concentrations. Two-dimensional LC methods for high-performance desalting and separation of complex samples are thus on-line coupled with ESI-MS for the identification and characterization of intact proteins.[20,21] However, it is common knowledge that undesired interaction between proteins and LC stationary phases can generally cause protein adsorption or entanglement, which reduces protein recovery and separation. This is known to hold true in RP HPLC of very lipophilic, glycosylated, or high molar mass (>100 kDa) proteins,[22] and it represents particularly serious constraints in the case of low-abundance proteins. Increase in separation performance can be achieved by using low-sized particle packing and narrow-bore, long columns under high- or ultra-high-pressure conditions.[23] Upon these pressure conditions, however, protein degradation may occur. Organic modifiers used in RP HPLC mobile phases can also induce protein denaturation.[24] Although denaturation under controlled conditions can be part of the overall analytical procedure for intact protein characterization by ESI-MS, in most cases, maintenance of the three-dimensional, native structure in fact constitutes a necessary requirement in functional proteomics. Variation in structure flexibility (e.g., the so-called "molten globule" case), stability (as a consequence, for instance, of folding/unfolding or turnover), and morphology of proteins, however, reflect in changes in protein diffusion coefficient without changes in protein molar mass. Even though comprehensive LC and CZE are high-resolution methods, they are not particularly selective with respect to changes in the protein diffusion coefficient, which can in fact reflect differences not only in molar mass but also in protein morphology.

Field-flow fractionation (FFF) is a family of flow-assisted separation techniques that applies to the analysis of macromolecular and supramolecular analytes, including proteins.[25] Flow FFF (FlFFF) is the FFF variant that employs a secondary flow of mobile phase as the perpendicular field.[26] The driving force that structures separation in FlFFF is the viscous force exerted on

(9) Low, L. Y.; Hernandez, H.; Robinson, C. V.; O'Brien, R.; Grossmann, J. G.; Ladbury, J. E.; Luisi, B. *J. Mol. Biol.* **2002**, *319*, 87−106.
(10) Liang, Y.; Du, F.; Sanglier, S.; Zhou, B. R.; Xia, Y.; Van Dorsselaer, A.; Maechling, C.; Kilhoffer, M. C.; Haiech, J. *J. Biol. Chem.* **2003**, *278*, 30098−30105.
(11) Whitehouse, C. M.; Dreyer, R. N.; Yamashita, M.; Fenn, J. B. *Anal. Chem.* **1985**, *57*, 675−679.
(12) Olivares, J. A.; Nguyen, N. T.; Udseth, Y. C. R.; Smith, R. D. *Anal. Chem.* **1987**, *59*, 1230−1232.
(13) Hofstadler, S. A.; Swanek, F. D.; Gale, D. C.; Ewing, A. G.; Smith, R. D. *Anal. Chem.* **1995**, *67*, 1477−1480.
(14) Li, N.; Cook, L.; Santos, C.; Cass, C. E.; Mackey, J. R.; Dovichi, N. J. *Anal. Chem.* **2002**, *74*, 2573−2577.
(15) Hu, S.; Zhang, L.; Krylov, S.; Dovichi, N. J. *Anal. Chem.* **2003**, *75*, 3495−3501.
(16) Cao, P.; Moini, M. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 184−186.
(17) Rochu, D.; Pernet, T.; Renault, F.; Bon, C.; Masson, P. *J. Chromatogr., A* **2001**, *910*, 347−357.
(18) Brenner-Weiss, G.; Kirschhofer, F.; Kuhl, B.; Nusser, M.; Obst, U. *J. Chromatogr., A* **2003**, *1009*, 147−153.
(19) Corradini, D.; Huber, C. G.; Timperio, A. M.; Zolla, L. *J. Chromatogr., A* **2000**, *886*, 111−121.
(20) Opiteck, G. J.; Lewis, K. C.; Jorgenson, J. W.; Anderegg, R. J. *Anal. Chem.* **1997**, *69*, 1518−1524.
(21) Opiteck, L. G.; Ramirez, S. M.; Jorgenson, J. W.; Moseley, M. A., III. *Anal. Biochem.* **1998**, *349*, 349−361.
(22) Nugent, K. D. In *High-Performance Chromatography of Peptides and Proteins*; Mant, C. T., Hodges, R. S., Eds.; CRC Press: Boca Raton, FL, 1991; Vol. 1, pp 279−287.
(23) Tolley, L.; Jorgenson, J. W.; Moseley, M. A. *Anal. Chem.* **2001**, *73*, 2985−2991.
(24) Wilson, K.; Walker, J. *Principles and techniques of practical biochemistry*, 5th ed.; Cambridge University Press: Cambridge, U.K., 2000.
(25) Giddings, J. C. In *Field-Flow Fractionation Handbook*; Schimpf, M. E., Caldwell, K. D., Giddings, J. C., Eds.; Wiley-Interscience: New York, 2000.
(26) Ratanathanawongs-Williams, S. K. In *Field-Flow Fractionation Handbook*; Schimpf, M. E., Caldwell, K. D., Giddings, J. C., Eds.; Wiley-Interscience: New York, 2000; Chapter 17.

sample components by the cross-flow stream. For this reason, retention time in FlFFF is, in principle, proportional to the analyte diffusion coefficient.[27,28] FlFFF shows intrinsic advantages for the analysis of intact proteins in native form. First, there is very little (if any) mechanical or shear stress on the protein molecule since the separation channel is empty. This allows for separation of high molar mass proteins and protein complexes without analyte entanglement/adsorption on the stationary phase. Second, FlFFF is so versatile that almost any liquid solution can be used as mobile phase. This is a key point to avoid possible protein degradation due to the mobile phase and to find the most compatible mobile phase for ESI. Third, FlFFF selectivity in terms of differences in diffusion coefficient is particularly high. Fourth, sample contaminants of low molar mass such as salts are not retained due to the porous channel walls. These features make FlFFF able to purify and separate intact protein samples within a broad molar mass range according to even very small differences in diffusion coefficient. Being differences in diffusion coefficient induced by differences in protein hydrodynamic radius, differences in FlFFF retention can indicate differences not only in molar mass but also in protein conformation. ESI/TOFMS analysis can then establish whether retention differences are a consequence of differences in molar mass or not. ESI/TOFMS characterization of intact proteins in their native form may be, then, significantly enhanced through on-line FlFFF. To authors' knowledge, however, only one and very preliminary study on on-line FlFFF-ESI-MS is described in the literature.[29] In that report, only the case of synthetic polymers of relatively low molar mass was considered, and no application to proteins was reported. Moreover, that study brought light on three drawbacks able to limit effective use of on-line FlFFF for ESI/TOFMS of proteins. First, a relatively high sample dilution occurs at the FlFFF outlet. Second, since relatively low flow rate conditions are needed at the ESI source inlet to reach high sensitivity, high flow splitting ratios should be required at the FlFFF channel outlet should be required. These factors can increase the limit of detection of proteins by current ESI/TOFMS methods. This is a particularly serious constraint in the case of low-ionized or low-abundance proteins. Third, possible run-to-run sample carryover due to incomplete sample recovery after FlFFF can contaminate spectra, thus affecting protein identification capabilities of ESI/TOFMS.

The idea of using hollow-fiber (HF) membranes as cylindrical, microcolumn channels for FlFFF (HF FlFFF) dates back to 1974,[30] with fundamentals on HF FlFFF given only in the late 1980s.[31,32] The ability of HF FlFFF to separate water-soluble polymers,[33,34] synthetic organic-soluble polymers,[35] and nanosized particles [36,37] based on differences in their diffusion coefficient was further described. We have recently shown that a wide selection of HF membranes enables HF FlFFF to separate micrometer-sized particles,[38] and particles of biological origin such as whole bacteria, yeast, and red blood cells,[39,40] due to differences in their physical characteristics. Most recently we have reported improvements in matrix-assisted, laser desorption/ionization (MALDI) TOFMS analysis of whole bacteria through off-line HF FlFFF.[41] Fractionation performance and sample recovery of our HF FlFFF version is nowadays comparable to that of commercial, flat-channel FlFFF systems. With respect to commercial FlFFF, however, our HF FlFFF version shows unique, intrinsic features for on-line coupling to ESI/TOFMS: (a) low channel volume (in the order of 100 μL), which reduces sample dilution; (b) low flow rate conditions (as low as 400 μL/min), which does not require high split ratios between the HF FlFFF outlet and the ESI source; (c) possible disposable usage, which eliminates the risk of run-to-run sample carryover and, then, spectra contamination. Incidentally, features (a) and (b) can decrease the limit of detection in ESI/TOFMS of low-abundance proteins, while feature (c) is also essential to reduce biohazards. Because of the hollow-fiber porosity, HF FlFFF can also give in-line sample micropurification, due to in-channel sample focusing/relaxation before the elution.[41]

In this work, we propose for the first time on-line HF FlFFF-ESI/TOFMS for the analysis of intact proteins. Proteins within a broad molar mass range (from horse heart myoglobin to horse spleen ferritin) are differently retained in the HF FlFFF channel according to differences in their diffusion coefficient. It is then demonstrated that the native (or pseudonative) characteristics of some standard proteins and enzymes such as horse heart myoglobin and horseradish peroxidase are maintained through the HF FlFFF process, with reduction of adduct ion formation due to in-line sample desalting. By relating the protein molar mass values derived from the diffusion coefficient values measured through HF FlFFF to the nominal and to the experimental molar mass values that are independently obtained through ESI/TOFMS, aggregation features and possible changes in the quaternary structure are obtained in the case of bovine serum albumin and human hemoglobin, respectively.

EXPERIMENTAL SECTION

HF FlFFF. The employed HF FlFFF system was a trial prototype, and the HF FlFFF channels were not commercially available. The channel was homemade of a piece of hollow fiber sheathed by two pieces of $^1/_8$-in. o.d. Teflon tube.[36,41] Two types of polysulfone hollow fibers were used. Inner radius and molar mass cutoff values of the used hollow fibers are listed in Table 1. Channel length was always 24 cm.

HF FlFFF operations with disposable channels typically involve three steps.[41] First, a makeup run is performed with an aliquot of the sample to condition the hollow-fiber membrane of the new

(27) Liu, M. K.; Li, P.; Giddings, J. C. *Protein Sci.* **1993**, *2*, 1520−1531.
(28) Fuh, C. B.; Levin, S.; Giddings, J. C. *Anal. Biochem.* **1993**, *208*, 80−87.
(29) Hassellov, M.; Hulthe, G.; Lyven, B.; Stenhagen, G. *J. Liq. Chromatogr. Relat. Technol.* **1997**, *20*, 2843−2856.
(30) Lee, H.-L.; Reis, J. F. G.; Dohner, J.; Lightfoot, E. N. *AIChE J.* **1974**, *20*, 776−784.
(31) Carlshaf, A.; Jönsson, J. A. *J. Chromatogr.* **1989**, *461*, 89−93.
(32) Jönsson, J. A.; Carlshaf, A. *Anal. Chem.* **1989**, *61*, 11−18.
(33) Wijnhoven, J. E. G. J.; Koorn, J.-P.; Poppe, H.; Kok, W. Th. *J. Chromatogr., A* **1995**, *699*, 119−129.
(34) Wijnhoven, J. E. G. J.; Koorn, J.-P.; Poppe, H.; Kok, W. Th. *J. Chromatogr., A* **1996**, *732*, 307−315.
(35) van Bruijnsvoort, M.; Kok, W. Th.; Tijssen, R. *Anal. Chem.* **2001**, *73*, 4736−4742.
(36) Lee, W. J.; Min, B.-R.; Moon, M. H. *Anal. Chem.* **1999**, *71*, 3446−3452.
(37) Moon, M. H.; Lee, K. H.; Min, B.-R. *J. Microcolumn Sep.* **1999**, *11*, 676−681.
(38) Min, B.-R.; Kim, S. J.; Ahn, K.-H.; Moon, M. H. *J. Chromatogr., A* **2002**, *950*, 175−182.
(39) Reschiglian, P.; Roda, B.; Zattoni, A.; Min, B.-R.; Moon, M. H. *J. Sep. Sci.* **2002**, *25*, 490−498.
(40) Reschiglian, P.; Zattoni, A. Roda, B.; Cinque, L.; Melucci, D.; Min, B.-R.; Moon, M. H. *J. Chromatogr., A* **2003**, *985*, 519−529.
(41) Reschiglian, P.; Zattoni, A.; Cinque, L.; Roda, B.; Dal Piaz, F.; Roda, A.; Moon, M. H.; Min, B. R. *Anal. Chem.* **2004**, *76*, 2103−2111

Table 1. Hollow-Fiber Membranes Employed for the HF FlFFF Channels

HF inner radius (cm, dried conditions)	M_r cutoff	manufacturer
0.041	30 000	SKU, Korea
0.040	6000	Asahi Kasei, Japan

channel. Two further steps, which correspond to different flow rates and flow patterns, are then performed: (a) sample injection/focusing/relaxation and (b) channel elution. During (b), one HPLC pump generated the required flow rates, while for (a) a second, syringe pump was also used. The HPLC pump was a model 422 (Bio-Tek Kontron Instruments, Milan, Italy), and the syringe pump was a model Pump 11 (Harvard Bioscience, Holliston, MA). Flow pattern conversions were switched using four- and three-way switching valves (Hamilton, Reno, NV).[40,41] Sample injection was made via a model 7125 injection valve (Rheodyne, Cotati, CA) equipped with a 20-μL external PEEK loop. The sample injection/focusing/relaxation process then operated for 3−5 min, with the focusing point determined as previously described.[39] The flow patterns for the elution step were then set by turning off the syringe pump. These operations usually generate pressure pulses and variations of the mobile phase flow rate that are responsible for the transient peak sometimes observed at the beginning (void) of the UV/visible fractograms of either blank or sample runs.

The cross-flow rate value (V_{rad}) for the elution step was chosen to obtain the best compromise between resolution and analysis time: the higher the cross-flow rate, the higher resolution, and the longer the analysis time. The cross-flow rate was manually adjusted through an SS-SS2-VH Nupro metering valve (Nupro, Willoughby, OH) positioned at the waste, radial outlet. The longitudinal channel flow rate value (V_{out}) for the elution step was determined through trial-and-error optimization. In principle, the higher the elution flow rate, the lower the separation efficiency, the shorter the analysis time, and the higher the flow split ratio required at the ESI source to obtain the best sensitivity. V_{rad} and V_{out} as low as 0.4 mL/min generally allowed achieving the best compromise. The required V_{out} was obtained by setting the HPLC pump at the channel inlet flow rate value (V_{in}) corresponding to the sum of V_{rad} and V_{out}, and the actual V_{rad} and V_{out} were manually measured by buret and chronometer.

Spectrophotometric UV/visible detection was made by a model UV 6000 LP high-sensitivity diode array spectrophotometer (ThermoQuest, Austin, TX) equipped with a fiber-optic guided, light-pipe cell. The cell path length was measured with a spectroscopic standard, as described in previous work.[39] The result was 4.6 ± 0.3 cm.

The HF FlFFF system was connected to the ESI source through a piece of PEEK tube (20 cm, 0.005-in. i.d., $^1/_{16}$-in. o.d.; Upchurch Scientific, Oak Harbor, WA) and a low-volume (maximum nominal, 2.4 μL) microsplitter valve (Upchurch Scientific) positioned between the channel and the UV/visible detector. Qualitative comparison between UV/visible and TIC fractograms showed no significant differences in band broadening and between the retention time values taken in correspondence of the fractogram maximums. This indicated that the so-designed on-line

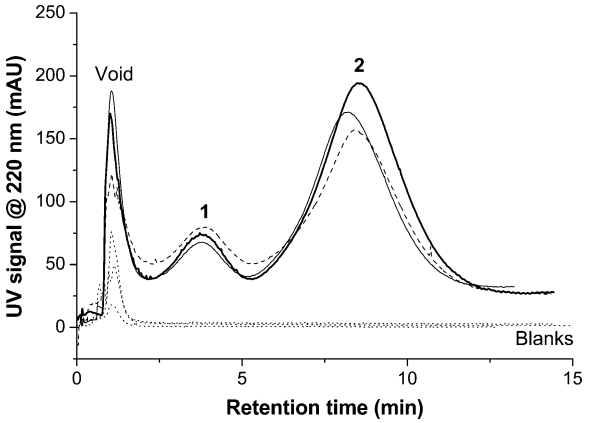

Figure 1. HF FlFFF of a binary mixture of whole proteins: (1) Mb 0.02% w/v; (2) BSA 0.05% w/v. V_{out} = 0.28 mL/min; V_{rad} = 0.42 mL/min. Three repeated runs.

connection between the HF FlFFF system, the UV/visible detector, and the ESI/TOF mass spectrometer did not give significant extracolumn effects. The split and waste flow streams were directly fed to the UV/visible detector cell and to the ESI source, respectively. During injection/focusing/relaxation, the ESI source was excluded from the flow circuit by a shut-off valve. The splitting valve thumbscrew allowed regulation of the stream flow rate ratio during elution. With V_{out} values around 0.3 mL/min, a split ratio of ~50:50 gave flow rate values at the ESI tip around 0.15 mL, which were found to give the best ESI/TOFMS sensitivity. It is known that the ESI/TOFMS response depends on the analyte concentration, and then, it should be independent of the flow rate at the source tip. However, ESI efficiency increases with decreasing flow rate. Flow rate values at the tip as low as 0.2 mL are commonly indicated as necessary to have enough sensitivity in ESI-MS coupled with flow-assisted separation techniques. The sketch diagram of the so-designed HF FlFFF-ESI/TOFMS system is separately reported as Supporting Information, Figure A.

The chosen mobile phase was a solution of 50 mM ammonium acetate (Sigma Aldrich, St. Louis, MO) at pH 7.0 in Milli-Q water (Millipore, Bedford, MA), which neither degraded proteins nor suppressed ionization. This mobile phase contained no metal ions to avoid metal/protein adduct formation during ESI. For the experiments performed with human hemoglobin at pH 8.2, reagent-grade ammonia (Sigma Aldrich) was added to the mobile phase. Proteins were dissolved in the mobile phase in a concentration range of 0.02−0.2% w/v. Protein samples were obtained from Sigma Aldrich or Boehringer Ingelheim (Ingelheim, Germany). The samples used in this work are listed in Table 2.

RP HPLC. A System Gold HPLC (Beckman Instruments, Fullerton, CA) was employed. The column was a Jupiter C4 (50 × 2.1 mm; Phenomenex, Torrance, CA). The mobile phase solutions were as follows: (A) 0.05% trifluoroacetic acid (TFA), 2% formic acid (FA) in Milli-Q water; (B) 0.05% TFA, 2% FA in acetonitrile. Gradient elution was performed from 30 to 65% in (B) in 20 min, at a flow rate of 0.20 mL/min.

ESI/TOFMS. A quadrupole-TOF hybrid mass analyzer, the model Micromass Q-TOF Micro (Micromass, Manchester, U.K.), was employed with a Z-spray ion source. The best ionization parameters were sought to minimize denaturation effects. The source temperature was set at 333 K, the capillary voltage at 2500

Table 2. Protein Samples

protein	M_r	source	catalog no.
myoglobin (Mb)	17 566.7[a]	horse heart	M-1882 (Sigma)
peroxidase (HRP)	42 382.7[b]	horseradish	P-6782 (Sigma)
hemoglobin (Hb)	63 218.2[c]	human	H-7379 (Sigma)
albumin (BSA)	66 398.6	bovine serum	A-2153 (Sigma)
alkaline phosphatase (AP)	157 204[d]	calf intestine	567744 (Boehringer)
ferritin (Fer)	440 000	horse spleen	F-4503 (Sigma)

[a] Including heme group. [b] From data in Figure 6b. [c] Including heme groups. [d] Peptide chain only.

V, the cone voltage at 30 V, and the collision energy at 5 V. Mass spectra were acquired in a m/z range spanning 800−3000 Thompson, and mass calibration over the entire range was performed by means of direct injections of egg lysozyme (Sigma Aldrich). Spectra were elaborated by Mass Lynks (Waters Corp., Milford, MA).

RESULTS AND DISCUSSION

Fractionation of Proteins. 1. Retention. A systematic, experimental study on protein HF FlFFF has not as yet been described. According to the HF FlFFF retention theory,[35,36] the diffusion coefficient (D) and retention time (t_r) of proteins can be related as follows:

$$t_r = \frac{r_f^2}{8D} \ln \frac{V_{in}}{V_{out}} \quad (1)$$

where r_f is the hollow-fiber inner radius. Protein D can be expressed as

$$D = kT/3\pi\eta d \quad (2)$$

where η is the mobile-phase viscosity, T the absolute temperature, k the Boltzmann constant, and d the protein hydrodynamic radius. Substituting eq 2 into eq 1, protein d values can be determined from the t_r values. According to a hard-sphere model, protein d increases with increasing the molar mass. In HF FlFFF, proteins of larger molar mass should then be eluted later than proteins of smaller molar mass.

2. Resolution, Reproducibility, and Recovery. Figure 1 shows the HF FlFFF separation of a mixture of horse heart myoglobin (Mb; nominal M_r = 17 566.7) and bovine serum albumin (BSA; nominal M_r = 66 398.6). Resolution between the two proteins appears to be relatively poor, particularly if compared to typical RP HPLC resolution. Bands are indeed relatively broad. This is however a general fact in FFF, because the sample polydispersity contribution to FFF band broadening is not destructive to separation but it reflects the high FFF selectivity.[42] Like conventional, flat-channel FlFFF, HF FlFFF is highly selective in terms of differences in D. High D-based selectivity then gives relatively broad peaks. As a consequence, HF FlFFF is in principle able to fractionate intact proteins according to sample heterogeneities that reflect into small differences in D. This makes particularly interesting use of HF FlFFF with ESI/TOFMS since whole proteins or protein complexes can be fractionated in terms of differences in diffusion coefficient, and the molar mass can be independently determined through ESI/TOFMS.

Since early developments, separation reproducibility and sample recovery were major concerns in HF FlFFF. In our previous work with bacterial cells, we demonstrated good run-to-run HF FlFFF reproducibility.[39] The use of disposable HF FlFFF channels indeed eliminates the risk of run-to-run sample carryover due to poor sample recovery. However, even with disposable fractionators, high levels of proportionate recovery are necessary. In fact, proportionate recovery reflects recovery of the different sample components in amounts proportional to their absolute amounts in the sample. High proportionate recovery has been shown in previous work on HF FlFFF for MALDI/TOFMS of whole bacteria.[41] High proportionate recovery levels can enhance also HF FlFFF-ESI/TOFMS analysis of whole proteins. In Figure 1 are superimposed the fractograms obtained from three different, consecutive runs of the protein mixture. The run-to-run reproducibility is acceptable, in terms either of resolution or of proportionate recovery, if it is considered that the runs were performed after several runs of the sample with the same channel under the same experimental conditions. As it can be deduced from the blank runs performed after each run, neither interrun carryover effects are shown.

3. Fractionation Performance. Figure 2a shows the superimposed UV traces of the fractograms of five single proteins: (1) Mb; (2) horseradish peroxidase (HRP); (3) BSA; (4) calf intestinal alkaline phosphatase (AP); (5) horse spleen ferritin (Fer). Each fractogram was individually obtained at the same V_{out} and V_{rad}. The results show, first, that higher molar mass proteins are more retained than lower molar mass proteins. Second, HF FlFFF can be successfully applied to distinguish whole proteins, including very high molar mass proteins (see the case of Fer; nominal M_r = 440 000), in a broad molar mass range: it is known, for instance, that SEC dramatically loses molar mass-based selectivity when applied to high molar mass proteins, because of shear degradation. Third, it can be noted that Mb, HRP, and AP fractograms appear to be monomodal (bands 1, 2, and 4, respectively), while the BSA and Fer fractograms appear to be bimodal (bands 3 and 3′) and trimodal (bands 5, 5′, and 5″), respectively. The secondary bands observed in the BSA and Fer fractogram could be due to protein aggregates. Figure 2b reports the UV traces of the fractograms obtained when BSA is treated with 9 M urea for 10 days. The intensity of the more retained bands increases by adding urea to the BSA sample (2′ and 3′ vs 1′), and the effect is more pronounced by increasing the sample stock temperature (3′ vs

(42) Davis, J. M. In *Field-Flow Fractionation Handbook*; Schimpf, M. E., Caldwell, K. D., Giddings, J. C., Eds.; Wiley-Interscience: New York, 2000; Chapter 3.

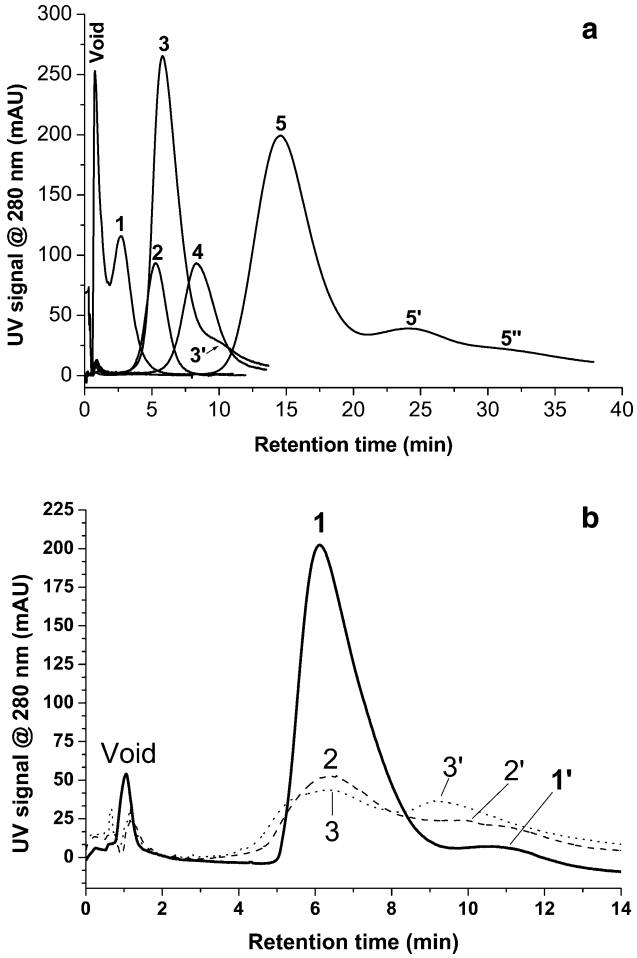

Figure 2. HF FlFFF of intact proteins. (a) Fractionation of single proteins: (1) 0.1% w/v Mb; (2) 0.1% w/v HRP; (3, 3′) 0.2% w/v BSA; (4) 0.1% w/v AP; (5, 5′, 5″) 0.1% w/v Fer. V_{out} = 0.32 mL/min; V_{rad} = 0.38 mL/min. (b) Fractionation of denatured BSA: (1,1′, full line) 0.2% w/v BSA, 298 K; (2, 2′, dashed line) 0.2% w/v BSA in 9 M urea, 277 K; (3, 3′, dotted line) 0.2% w/v BSA in 9 M urea, 298 K. V_{out} = 0.32 mL/min; V_{rad} = 0.38 mL/min.

2′). It is known that when BSA is in solution with highly concentrated urea, hydrophobic interactions increase. If the urea-treated BSA sample is injected into the HF FlFFF system with ammonium acetate as mobile phase, during the focusing/relaxation step inside the HF FlFFF channel, the BSA molecules reach a relatively high local concentration in a solvent that is more polar than when urea is present in the batch sample. As a consequence, before the elution, the BSA molecules tend to maximize intermolecular hydrophobic interactions, thus increasing formation of protein aggregates that are eluted at higher retention time.

Figure 3 reports the fractograms obtained with human hemoglobin (Hb) at mobile-phase pH 7.0 and pH 8.2. An incident wavelength of 420 nm was used because it corresponds to Hb's highest absorbance. The Hb fractogram obtained at pH 8.2 (fractogram 2) is shifted toward a retention time that is higher than at pH 7 (fractogram 1). According to the HF FlFFF retention theory (eq 1), this retention difference at a different pH is due to a change in the protein diffusion coefficient, which can be related to a change in the protein hydrodynamic radius (eq 2) that can reflect changes in either molar mass or protein conformation.

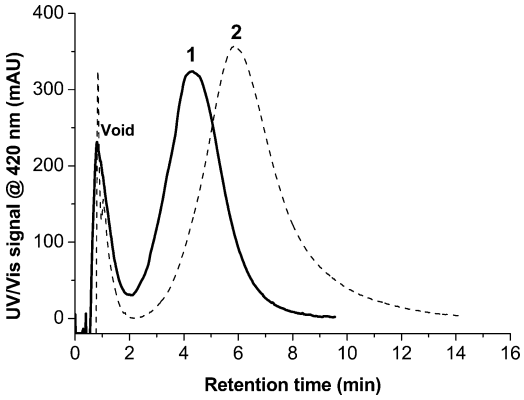

Figure 3. HF FlFFF of Hb at different pH. Sample concentration, 0.1% w/v. (1) pH = 7.0; (2) pH = 8.2. V_{out} = 0.32 mL/min; V_{rad} = 0.38 mL/min.

Confirmation of possible aggregate formation or of structural changes that reflect into fractogram features may be then given by ESI/TOFMS analysis, as will be discussed in BSA Aggregation and pH-Dependent Behavior of Hb.

4. Maintenance of Native Conditions. (a) HF FlFFF-ESI/TOFMS versus RP HPLC-ESI/TOFMS of Mb. In Figure 4, the multicharged ion spectra obtained through HF FlFFF-ESI/TOFMS (a) and RP HPLC-ESI/TOFMS (b) of Mb are reported. Slightly different protonation levels can be observed. In the case of RP HPLC-ESI/TOFMS, Mb seems to incorporate a slightly higher number of protons through ESI, which gives rise to a shift of the ion distribution pattern to lower m/z values. This slightly higher number of acquired protons could bear the first indications of possible Mb denaturation through RP HPLC. From the multicharge ion spectra reported in Figure 4, the mass spectra were derived (not shown). In the case of RP HPLC-ESI/TOFMS, one species with M_r = 16 951.5 ± 0.27 was found. This value agrees well with the nominal molar mass of apomyoglobin (M_r = 16 951.5), that is, of Mb without the heme group the molar mass of which is M_r = 615.23. Nonetheless, through HF FlFFF-ESI/TOFMS, a single species of M_r = 17 566.7 ± 0.6 was observed. This molar mass value is not significantly different from the nominal mass of intact Mb. These findings prove that when it is separated through HF FlFFF rather through RP HPLC, Mb mostly maintains the noncovalent bond between the polypeptide chain and the heme group. Degradation of Mb through RP HPLC could occur either because of the low pH, the presence of an organic modifier in the mobile phase, or the relatively hydrophobic stationary phase. When Mb was dissolved in the mobile phase, and directly injected into the ESI-MS without previous RP HPLC separation, identical results were in fact obtained. This indicates that when Mb was eluted through RP HPLC the mobile-phase composition was the factor able to degrade Mb.

Desalting. In-line sample desalting and cleanup through hollow-fiber microdialysis was successful for ESI-MS analysis of intact proteins and nucleic acids.[43−45] The microdialysis systems

(43) Wu, Q.; Liu, C.; Smith, R. D. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 835−838.
(44) Liu, C.; Wu, Q.; Harms, A. C.; Smith, R. D. *Anal. Chem.* **1996**, *68*, 3295−3299.
(45) Liu, C.; Hofstadler, S. A.; Bresson, J. A.; Udseth, H. R.; Tsukuda, T.; Smith, R. D.; Synder, A. P. *Anal. Chem.* **1998**, *70*, 1797−1801.

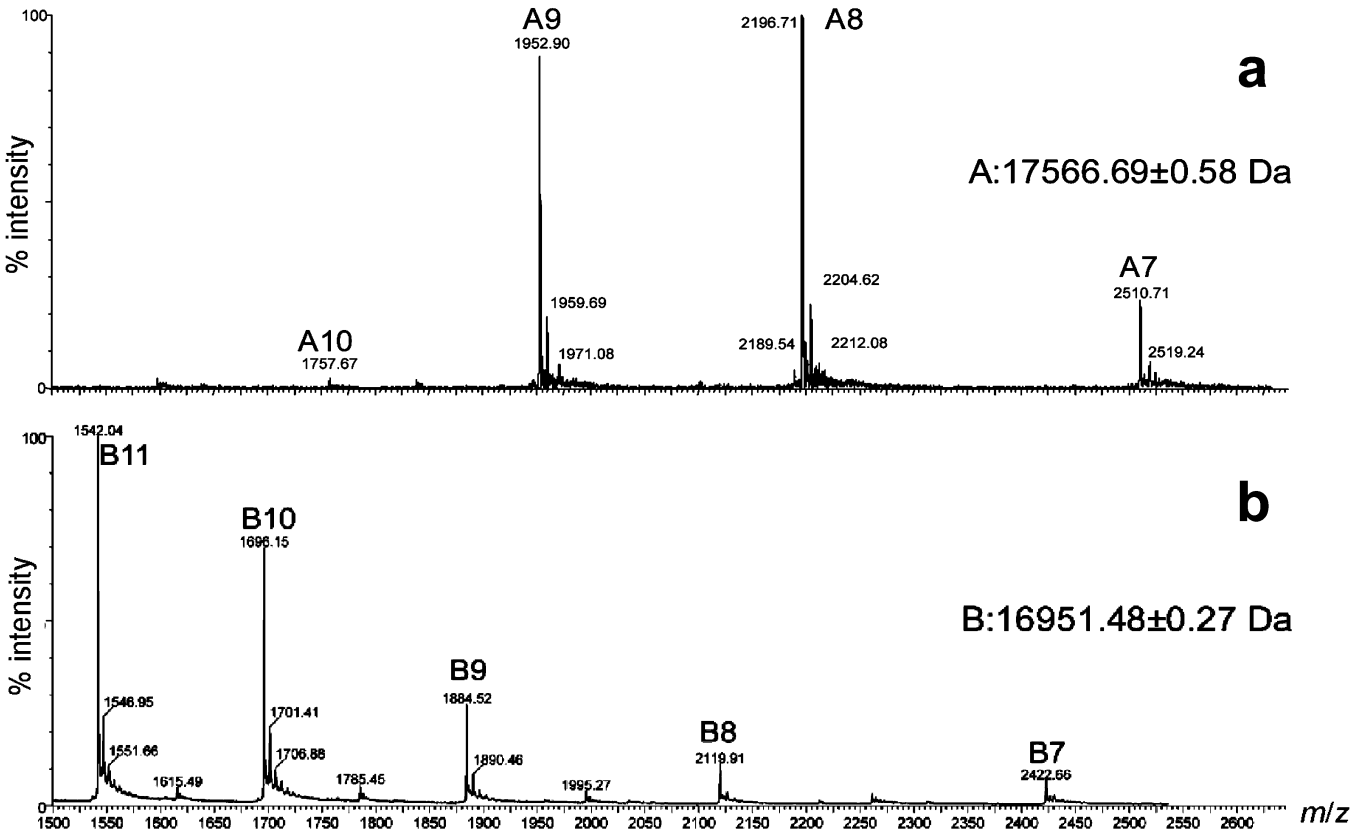

Figure 4. Comparison between HF FlFFF-ESI/TOFMS and RP HPLC-ESI/TOFMS of Mb: (a) multicharged ion spectrum from HF FlFFF-ESI/TOFMS; (b) multicharged ion spectrum from RP HPLC-ESI/TOFMS.

were designed by connecting a home-built microdialysis cartridge to the ESI source, with the sample continuously flowing into the inner side and the dialysis buffer flowing toward the outer side of the hollow fiber. Being designed for microdialysis and not to exploit the FlFFF mechanism, the system did not in fact separate the analytes from narrow pulses of injected samples. In our HF FlFFF system, we can instead simultaneously obtain in-line separation and cleanup of the sample, as we have observed in HF FlFFF for MALDI/TOFMS of whole bacteria.[41] This is because the low molar mass species present in the sample plug injected into the HF FlFFF channel can be washed out from the inner to the radial exit of the channel during the sample relaxation/focusing step before elution of the higher molar mass species. Figure 5 compares the expanded view of the $(M + 9H^+)$ state of the spectrum obtained through direct ESI/TOFMS (a) and through HF FlFFF-ESI/TOFMS (b) of the Mb sample. In both cases, the Mb sample batch was diluted in 50 mM ammonium acetate without any type of sample treatment before the analysis. Significant differences can be observed between the spectra. In the case of direct ESI/TOFMS (Figure 5a), many Na^+/Mb adducts are present, while it is not the case for HF FlFFF-ESI/TOFMS (Figure 5b), where only a low-intensity signal due to the CH_3COO^-/Mb adduct is found. As described above, the molar mass measured from the derived HF FlFFF-ESI/TOF mass spectrum gave the molar mass of intact Mb. Although a systematic study on sample desalting through HF FlFFF stands beyond the aims of the present work, these preliminary findings indicate that HF FlFFF can give desalting without protein degradation while proteins are fractionated according to their diffusion coefficient values. This should represent a significant improvement in reducing the procedural complexity if HF FlFFF wanted to be included as a purification/separation step within a multidimensional approach to MS analysis of intact proteins.

(b) HF FlFFF-ESI/TOFMS of HRP. Figure 6 reports the multicharged ion spectrum (a) and the derived mass spectrum (b) obtained in correspondence to the HRP fractogram maximum reported in Figure 2a, case 2. HRP is an extracellular heme enzyme consisting of 308 amino acids and a single protoporphyrin IX prosthetic group, two calcium ions, four disulfide bridges, and three N-glycosylation sites.[46−48] RP HPLC-ESI/TOFMS of HRP revealed the presence of three species, the mass of which corresponded to the binding of, respectively, one, two, and three $(GlucNAc)_2-(Man)_3$ structures to the HRP polypeptide chain (data not shown). This oligosaccharide structure, which constitutes the common core of all the glycosidic anchors present in N-glycosylated proteins, has a nominal $M_r = 894$. In fact, when HRP was analyzed through HF FlFFF-ESI/TOFMS (Figure 6b), the molar mass of each of the three glycosylated species increased of ~615 Da with respect to the values observed in RP HPLC-ESI/TOFMS. The mass spectrum in Figure 6b also shows that the glycosylated species were present as doublets, while RP HPLC-ESI/TOFMS gave no mass signal doublets in correspondence to these species. Since the nominal molar mass of the heme prosthetic group is

(46) Morishima, I.; Kurono, M.; Shiro, Y. *J. Biol. Chem.* **1986**, *261*, 9391−9399.
(47) Dunford, H. B. In *Peroxidases in Chemistry and Biology*; Everse, J. E., Everse, K. E., Grisham, M. B., Eds.; CRC Press: Boca Raton, FL, 1991; pp 1−24.
(48) Yang, B. Y.; Gray, J. S.; Montgomery, R. *Carbohydr. Res.* **1996**, *287*, 203−212.

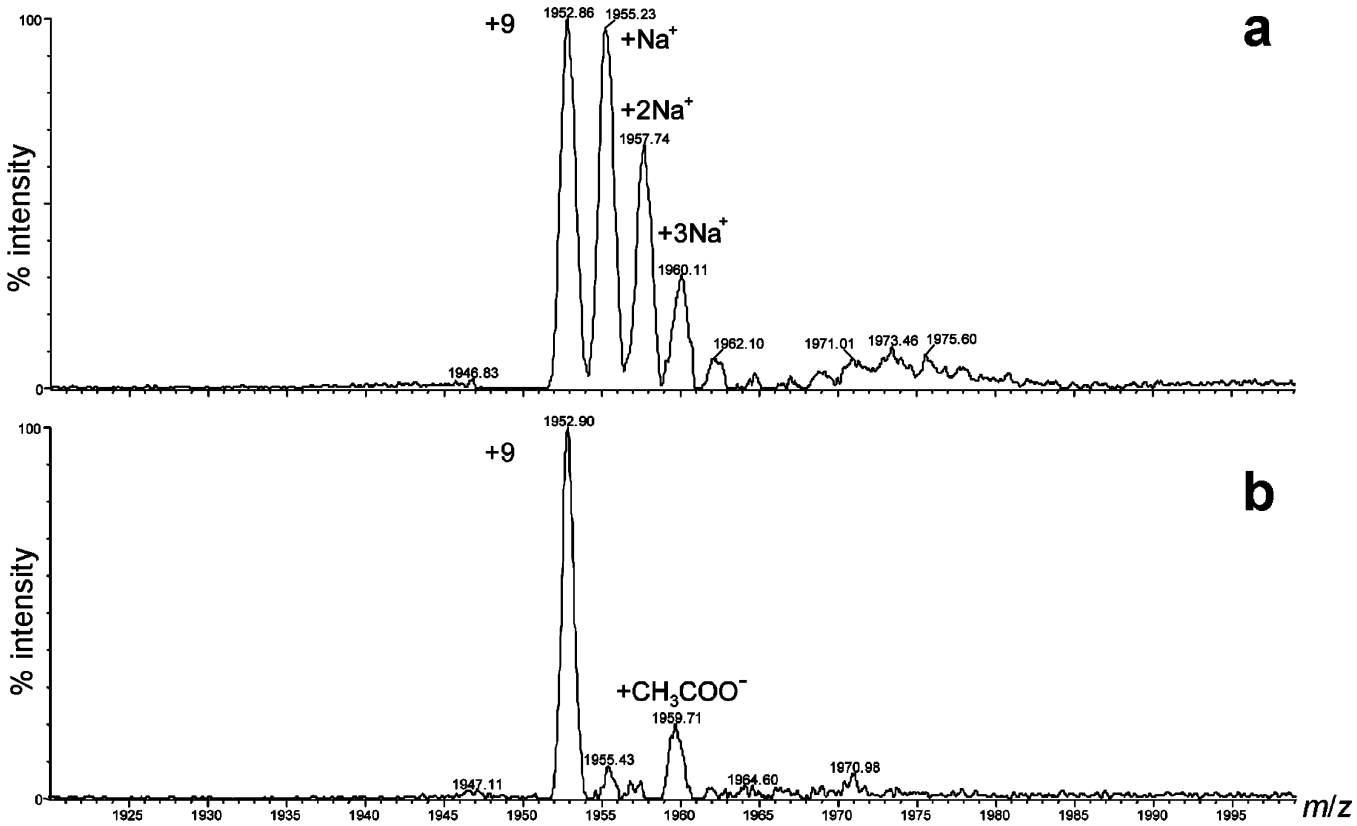

Figure 5. In-line sample desalting. Sample, Mb 0.2% w/v: (a) multicharged ion spectrum from direct ESI/TOFMS; (b) multicharged ion spectrum from HF FlFFF-ESI/TOFMS.

M_r = 615.23, and the difference in mass between the peaks of each doublet is ~80 Da, which is the mass of $2Ca^{2+}$, these findings support two important indications. First, as in the Mb case discussed in HF FlFFF-ESI/TOFMS versus RP HPLC-ESI/TOFMS of Mb above, the elution through HF FlFFF does not alter the noncovalent bond between the polypeptide chain and the prosthetic group as it does elution through RP HPLC. Second, comparing the relative intensity of the two peaks of each doublet, it is proved that most of the HRP molecules retain the calcium ions that are noncovalently bound to the enzyme. It is also worth noting the total absence of species corresponding to the binding of one, rather than two, calcium ion. This indicates the strong interdependence of the two calcium-binding sites, and it suggests that the observed species should not be generated by unspecific absorption of metal ions from the sample solution, as in the case of the unspecific absorption of Na^+ to Mb discussed above in Desalting.

Characterization of Proteins. To determine the molar mass value of proteins from HF FlFFF, a regression analysis is required. This is based on the correlation between the D values measured from retention times and the nominal M_r values [49]

$$D = AM_r^{-b} \tag{3}$$

where A and b are constants. If eq 3 is experimentally verified with protein standards, the M_r values of the unknowns can be obtained from the diffusion coefficient values (d) determined from the measured t_r values (eqs 1 and 2) and compared to the M_r values measured from the mass spectra obtained in correspondence with the t_r values. The experimental log D values from the t_r values taken in correspondence with the main peaks of the protein fractograms were plotted versus the nominal log M_r values. The regression plot is separately reported as Supporting Information, Figure B. The experimental log D values linearly decreased with increasing nominal log M_r values. Linear correlation was good (R^2 = 0.94; intercept, −4.5 ± 0.2), and the slope value (0.37 ± 0.04) was close to $^1/_3$, as expected from eq 3 using the hard-sphere model to describe the protein hydrodynamic behavior in an FFF channel.[49] The poorer correlation of the data point obtained for Hb at pH 8.2 (indicated as Hb2 in Supporting Information, Figure B) with respect to the data point obtained for Hb at pH 7 (indicated as Hb1 in Supporting Information, Figure B) may indicate that, at pH 8.2, the retention time shift observed for Hb in fractogram 2 of Figure 3 could have been due to a change in either molar mass or conformation. The well-correlated data points for BSA and Fer were obtained for D values from the t_r values taken in correspondence with, respectively, bands 3 and 5 of the fractograms in Figure 2a. The secondary bands present in the BSA and Fer fractograms (bands 3′ and 5′, Figure 2a) must then correspond to species whose D values are different from those corresponding to the nominal specifications. Independent M_r determination through ESI/TOFMS should then indicate whether the change in Hb diffusion coefficient at pH 8.2 was due to a change either in protein molar mass or in conformation and whether, for instance, the secondary band in the BSA fractogram represented

(49) Li, P.; Hansen, M. In *Field-Flow Fractionation Handbook*; Schimpf, M. E., Caldwell, K. D., Giddings, J. C., Eds.; Wiley-Interscience: New York, 2000; Chapter 28.

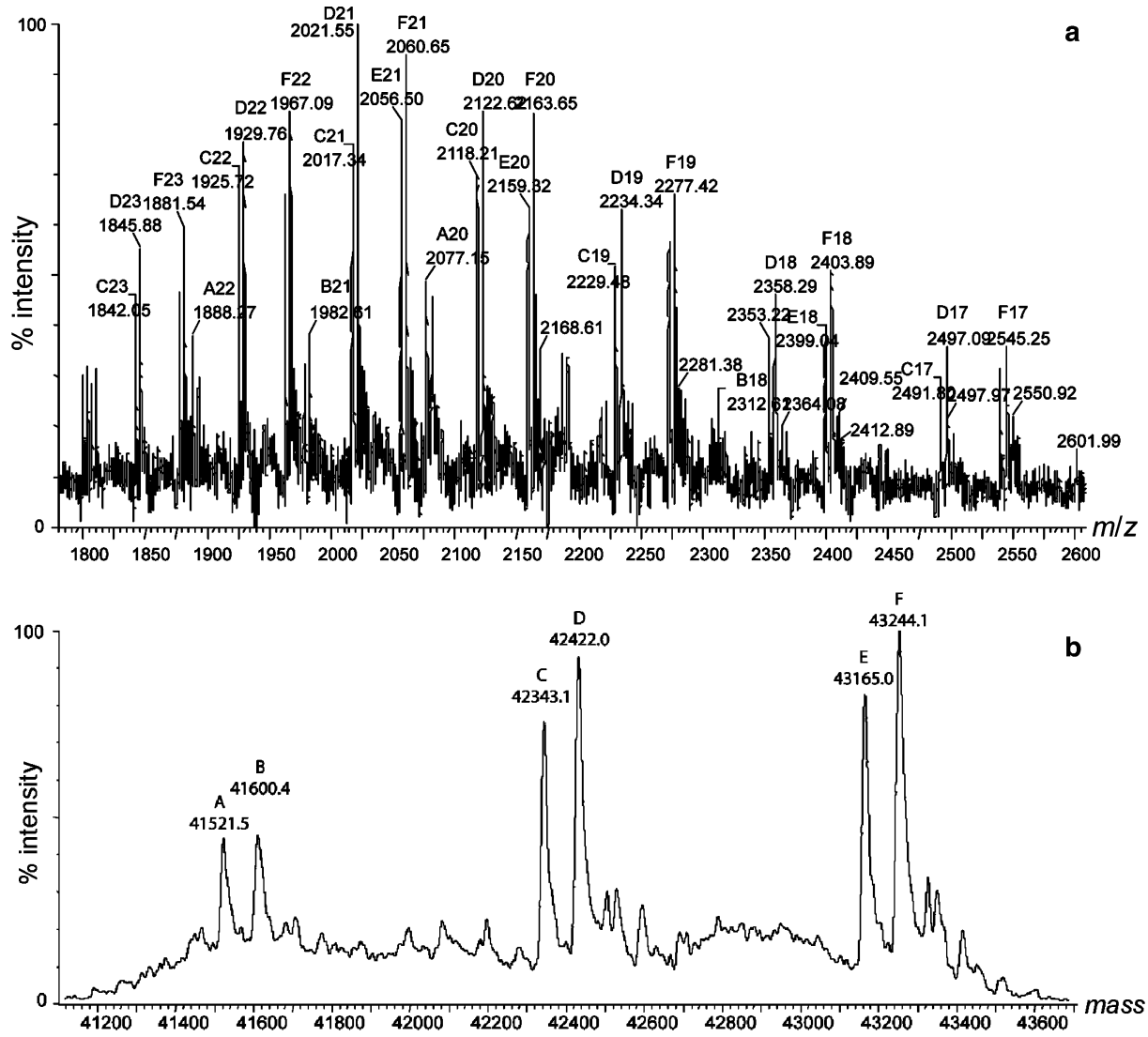

Figure 6. HF FlFFF-ESI/TOFMS of HRP: (a) multicharged ion spectrum; (b) molar mass spectrum.

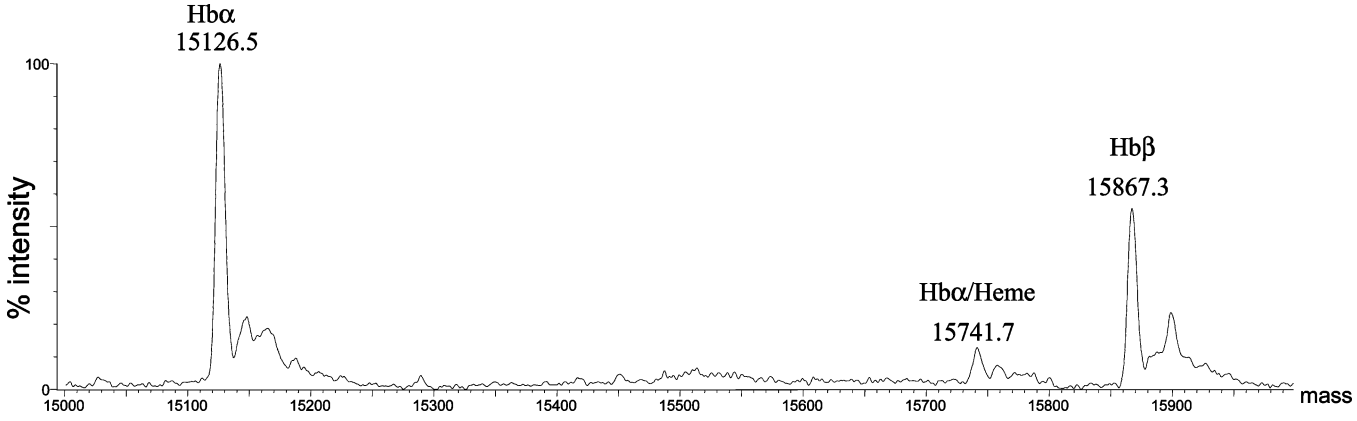

Figure 7. HF FlFFF-ESI/TOFMS of Hb: molar mass spectrum. V_{out} = 0.32 mL/min; V_{rad} = 0.38 mL/min.

a protein aggregate. It must be finally noted that the data employed for the regression analysis were obtained with two different HF FlFFF channels made up of membranes supplied by different manufacturers and of different molar mass cutoff: 30 (○) and 6 kDa (△). The log D values obtained using the two HF FlFFF channels were not significantly different. Incidentally, this supports channel-to-channel reproducibility of HF FlFFF of intact proteins.

1. BSA Aggregation. By means of the regression analysis described above, the M_r value obtained from the t_r value of band 3′ of the BSA fractogram of Figure 2a corresponds to the molar mass of the BSA dimer. The multicharge ion and mass spectra on-line recorded in correspondence with the t_r value of either band 3 or 3′ of the BSA fractogram in Figure 2a were, however, identical. They are separately reported as Supporting Information,

Figure C. The presence of just a single species of $M_r = 66\,398 \pm 2$ was assessed. This M_r value perfectly agrees with the nominal M_r of BSA. It is thus proved through independent techniques that band 3′ of the BSA fractogram in Figure 2a corresponds to the aggregate made up of two intact BSA units.

2. pH-Dependent Behavior of Hb. Native Hb is a tetramer constituted of two α and β subunits, each having one polypeptide chain and one heme group that is noncovalently bound to the subunit. In the literature, ESI-MS analysis of native Hb reports the molar mass values of the α and β subunits, since ESI breaks the noncovalent bonds between the tetramer subunits.[50] Figure 7 shows the mass spectrum obtained for HF FlFFF-ESI/TOFMS of Hb: identical spectra were obtained at pH 7 and 8.2. The mass spectrum shows the presence of three species, with M_r values corresponding to the M_r values of the α and β subunits ($M_r = 15\,126.5 \pm 0.3$, $M_r = 15\,867.3 \pm 0.5$, respectively), and of the α-heme complex ($M_r = 15\,741.5 \pm 0.7$). This corresponds to the spectrum of native Hb. The M_r value obtained from HF FlFFF retention of Hb at pH 7.0 (Figure 3, fractogram 1), however, corresponds to the M_r value of the tetramer. These findings suggest that, first, as in the case of Mb and HRP discussed in HF FlFFF-ESI/TOFMS versus RP HPLC-ESI/TOFMS of Mb and HF FlFFF-ESI/TOFMS of HRP above, the heme group is not released during HF FlFFF. In the literature it is reported that loss of the heme group in Hb results in globin chain unfolding and, then, decrease of protein stability and solubility.[51] This would have in fact affected HF FlFFF retention of Hb, with direct consequence on the fractogram profile. Second, the retention differences observed at different pH values could have been exclusively due to conformational changes of Hb as a function of pH. Hb is an allosteric protein; that is, it changes conformation when it interacts with other molecules. In Hb, oxygen molecules are bound to the heme group, and pH influences Hb affinity to oxygen (Bohr effect).[52] An increase of pH increases oxygen association,[53] which modifies the Hb quaternary structure. This can slightly change the Hb diffusion coefficient and then explain the observed change in retention by changing pH without apparent effects on the relevant spectra.

CONCLUSIONS

Compared to LC methods, HF FlFFF demonstrates the following interesting peculiarities when coupled to ESI/TOFMS for the analysis of intact proteins: (a) in-line sample desalting capabilities; (b) possibility of employing any mobile phase to (1) assess the best ESI compatibility and (2) minimize degrading conditions; (c) absence of stationary phase. Features (b) and (c) make the HF FlFFF retention mechanism particularly soft to fractionate proteins, even high molar mass proteins, based on differences in the protein diffusion coefficient, by preserving the noncovalent chemistry and then maintaining the fractionated proteins in their native (or pseudonative) form. Fractionation capabilities of our HF FlFFF version are equivalent to that of flat-channel, commercial FlFFF systems. However, for on-line coupling with ESI/TOFMS, HF FlFFF shows advantages such as possible disposable usage and low volume of the channel to (a) avoid run-to-run sample carryover, (b) reduce sample dilution and flow rate conditions, and (c) shorten separation time. Feature (b) can decrease the detection limits, which could be further decreased by using narrow-bore hollow fibers for nano HF FlFFF channels coupled with nanospray MS. Nano HF FlFFF may also increase separation efficiency. Low detection limits and high separation efficiency are key aspects in the identification and characterization of whole proteins present at low abundance in complex samples. Work is in progress to develop nano HF FlFFF. However, even though it is generally acknowledged that higher resolution should be required to improve HF FlFFF performance with complex samples, it must be noted that the high diffusion-based selectivity makes HF FlFFF intrinsically able to distinguish even small heterogeneities in terms of protein diffusion coefficient. In our opinion, this makes HF FlFFF-ESI/TOFMS unique in its ability to characterize intact proteins and possible changes in their structure as a consequence of either denaturation, aggregation, or interaction of the proteins with other species. We believe that HF FlFFF may be used as a complementary rather than an alternative separation technique of interesting potential when implemented in MS-based, multidimensional approaches to top-down or functional proteomics.

ACKNOWLEDGMENT

The authors are thankful to S. Casolari and D. Melucci, University of Bologna, for technical assistance. P.R. gratefully thanks M. Giddings, University of North Carolina, for careful manuscript revision and helpful suggestions. Grants: Work partially supported by the Italian Ministry of the Education, University and Research (MIUR, COFIN 2000, MM03247343-005) and by the University of Bologna, funds for selected research topics.

SUPPORTING INFORMATION AVAILABLE

Instrumental details (HF FlFFF-ESI/TOFMS system diagram) and additional results (regression plot and BSA spectra). This material is available free of charge via the Internet at http://pubs.acs.org.

(50) Hernan, R. A.; Sligar, S. G. *J. Biol. Chem.* **1995**, *270*, 26257−26264.
(51) Hrkal, Z.; Vodrázka, Z. *Biochim. Biophys. Acta* **1967**, *133*, 527−534.
(52) Kilmartin, J. V. *Br. Med. Bull.* **1976**, *32*, 209−212.
(53) Baldwin, J. M. *Br. Med. Bull.* **1976**, *32*, 213−218.

Received for review July 28, 2004. Accepted October 1, 2004.

AC048898O