Hollow-fiber flow field-flow fractionation: a novel pre-MS method for proteomics

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ABSTRACT

The development of new "pre-MS" methods for protein isolation/separation from complicated biological samples is a fundamental requirement for successful, MS-based approaches to proteomics. Flow field-flow fractionation (F4) has been applied as pre-MS step for proteomics. Hollow-fiber F4 (HF5) is the innovative, microcolumn version of F4. HF5 employs a piece of hollow fiber as separation channel and current HF5 shows performance comparable to commercial F4. The reduced channel volume and flowrates, the low dilution of fractionated analytes, and the potentially disposable usage to exclude contaminations or carryover then make HF5 ideally suited as online or offline pre-MS separation step for the characterization of complex protein samples in native form. We have developed different applications based on HF5 in combination with MS or LC-MS for the fractionation, purification and characterization of native proteins, and for proteomic analysis of complex protein samples.

Keywords: proteomics, pre-MS separation methods, hollow-fiber flow field-flow fractionation (HF5), native protein separation, subproteomics.

1 INTRODUCTION

It is widely acknowledged that, when mass spectrometry (MS) methods are applied to complex protein samples, the availability of so-called "pre-MS" methods is required when sample complexity exceeds the resolution capabilities of most sophisticated MS techniques. Many efforts are devoted, for instance, to mining the low-abundance proteins (LAP) among the huge wealth of high abundant proteins (HAP) possibly present in the sample.

MS with soft impact ion sources and time-of-flight (TOF) analyzers are the most applied techniques for bottom-up approaches to proteomics. Several studies have shown that biomarker identification is possible based on the presence/absence of multiple low-molar mass (low-M_r) serum components. However, the precise nature of the peptides contained in human serum, most of which are fragments of larger proteins, still remains largely unknown. On the other hand, few high-abundant proteins (HAP) represent most of the protein content in biological fluids, with thousands to millions of low-abundant proteins (LAP) that constitute a few percent in mass though they may span 10 orders of magnitude in relative concentration. These are the reasons for which most of the common approaches for clinical proteomics can show limitations related to the proteome composition and to the different protein expression levels, then giving method-dependent results.

1.1 Flow Field-Flow Fractionation

Among separation techniques, field-flow fractionation (FFF) has shown the broadest M_r application range. In the bio-analytical field, applications have been reported to span from proteins to whole cells [1, 2].

FFF uses an instrumental setup similar to liquid chromatography (LC). The separation run consists of the injection of a narrow sample band into a mobile phase stream that sweeps the sample components down the separation channel to finally reach a detector. The FFF mechanism is not based on interaction of the analyte with a stationary phase, but with an external field that is applied perpendicularly to the mobile phase flow. According to differences in their M_r, size, or other physical properties, the different analytes are driven by the orthogonal field into different velocity regions within the parabolic flow profile of the mobile phase across the channel.

Different field types have originated different FFF variants. In flow FFF (F4), the orthogonal field is a second stream of mobile phase that is applied across the channel (cross-flow). Due to the universality of the viscous force exerted on the analyte by the cross-flow stream, F4 is capable of separating macromolecules and particles from 1 nm to > 50 μm in size (e.g. from proteins to whole cells). The lower size limit is determined by the M_r cut-off of the accumulation wall, which is usually constituted of an ultrafiltration membrane able to retain the macromolecular analytes inside the channel. F4 has been applied as pre-MS step for proteomics [3]. Different F4 channel designs have been proposed: we can have flat channels, with the cross flow applied either in a symmetrical or asymmetrical (AF4) configuration, or tubular channels with a radial cross-flow configuration. In the latter case, a hollow-fiber (HF) membrane for micro-dialysis makes the accumulation wall of the channel (HF5) [4]

2 HOLLOW-FIBER FLOW FIELD-FLOW FRACTIONATION

Hollow-fiber F4 (HF5) is the innovative, ready-to-market, microscale version of F4 developed for MS

analysis of proteins and complex proteomes. Reduced channel volume and operation flow rates, low dilution of fractionated analytes make HF5 ideally suited for online and offline coupling with MS. Possible disposable usage allows to exclude contaminations or carryover when complex biological samples are analyzed. The HF5 device is made of a piece of cylindrical, polymeric HF membrane for microdialysis, with 10-30 kDa cutoff, sheathed by two pieces of Teflon tube. The HF5 channel is connected to a LC-like system and, in case of on-line HF5-MS, the outlet is connected to the ion source. In a typical HF5 arrangement, the HF channel is connected to a pump that generates a flow of mobile phase along the HF. A pressure drop between the inner and outer porous wall of the HF generates the radial cross-flow. Assuming laminar flow conditions, a parabolic flow profile is established in the HF.

When macromolecules (e.g. proteins) and relatively small (e.g. nanosized) particles are separated by HF5, the normal retention mode takes place. Sample components introduced in the HF channel undergo to a process of relaxation/focusing to achieve, before separation, a steady-state concentration profile across the HF section. During such a relaxation/focusing process, the migration of sample components towards the channel wall due to the radial flow generates a concentration gradient in the radial direction. Otherwise, sample particles diffuse in a direction opposite to the radial flow until they reach an equilibrium position. Sample components at different equilibrium distances from the HF wall are transported by the longitudinal flow at different velocities, hence they are eluted at different retention times.

A simplified expression, the retention time (t_r) is related to the HF inner radius R, the elution flow rates at the HF inlet and oulet (F_{in}, F_{out}) and the analyte diffusion coefficient D:

$$t_r = \frac{R^2}{8D} \ln\left(\frac{F_{in}}{F_{out}}\right) \tag{1}$$

A HF5 run typically involves two steps: sample injection/focusing/relaxation, and sample elution.

Injection is performed by introducing the sample in the HF channel using a longitudinal flow of mobile phase towards the HF channel outlet, and a second flow of mobile phase in opposite direction, from the outlet to the inlet of the HF channel. At the point where the resulting longitudinal flow rate is zero, said the focusing point, the injected analytes focus and achieve their steady-state condition (relaxation).

When the injection/focusing/relaxation process is completed, the system is set for the elution step: a single mobile phase flow stream is applied along the HF towards the channel outlet.

3 HF5 OF PROTEIN SAMPLES

For the analysis of intact proteins and protein complexes under native conditions HF5 shows additional features. Due to the absence of a stationary phase, the risk of altering the native protein conformation as a result of mechanical or shear stress on the analyte is very little (if any). As mobile phase, HF5 can utilize solutions of any composition and concentration. The mobile phases used with other separation techniques may cause alteration of protein three-dimensional conformations, or they may induce unintended dissociation of protein complexes during separation, or they can be incompatible with further MS characterization. Finally, since size-based selectivity of HF5 is particularly high, interaction/aggregation between fractionated proteins can be deduced from retention-based hydrodynamic size measurements.

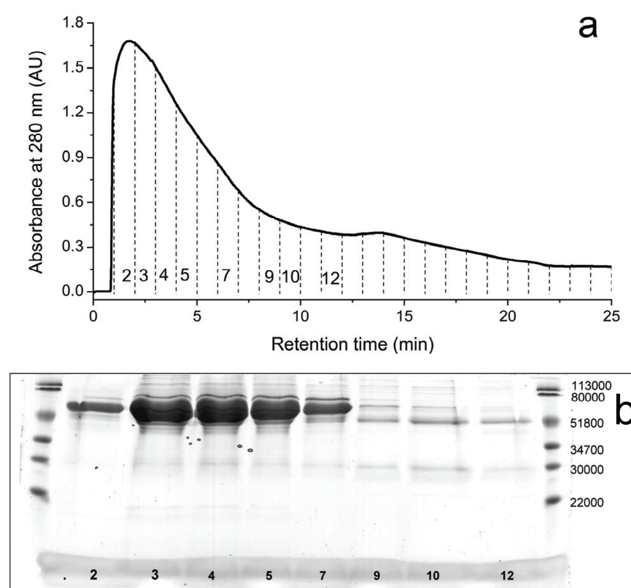

Figure 1. HF5 of human blood whole serum 1:5 v/v diluted in the mobile phase (5 mM NH_4Ac). (a) HF5 fractogram and fractions collected for SDS PAGE. (b) SDS PAGE of the collected fractions. PSf HF membrane: 30,000 M_r nominal cut-off, R = 0.040 cm (referred to dried conditions), L = 24 cm. Experimental conditions: F_{in} = 0.7 mL/min, F_{rad} = 0.4 mL/min. Reprinted with permission from ref. 6, © 2008, Elsevier Publishers.

The analysis of protein complexes is important, for instance, in studying functional drugs due to the relationship between self-association or dissociation phenomena and the protein drug activity. HF5 coupled with MALDI/TOF MS and with an enzyme activity assay with chemiluminescence detection has allowed relating the supramolecular structure of an enzyme drug (uricase) with its enzymatic activity [5].

HF5 shows to be interesting also as a pre-MS method to reduce complexity of biological fluids. For instance, when applied to untreated, whole human blood serum, HF5 is

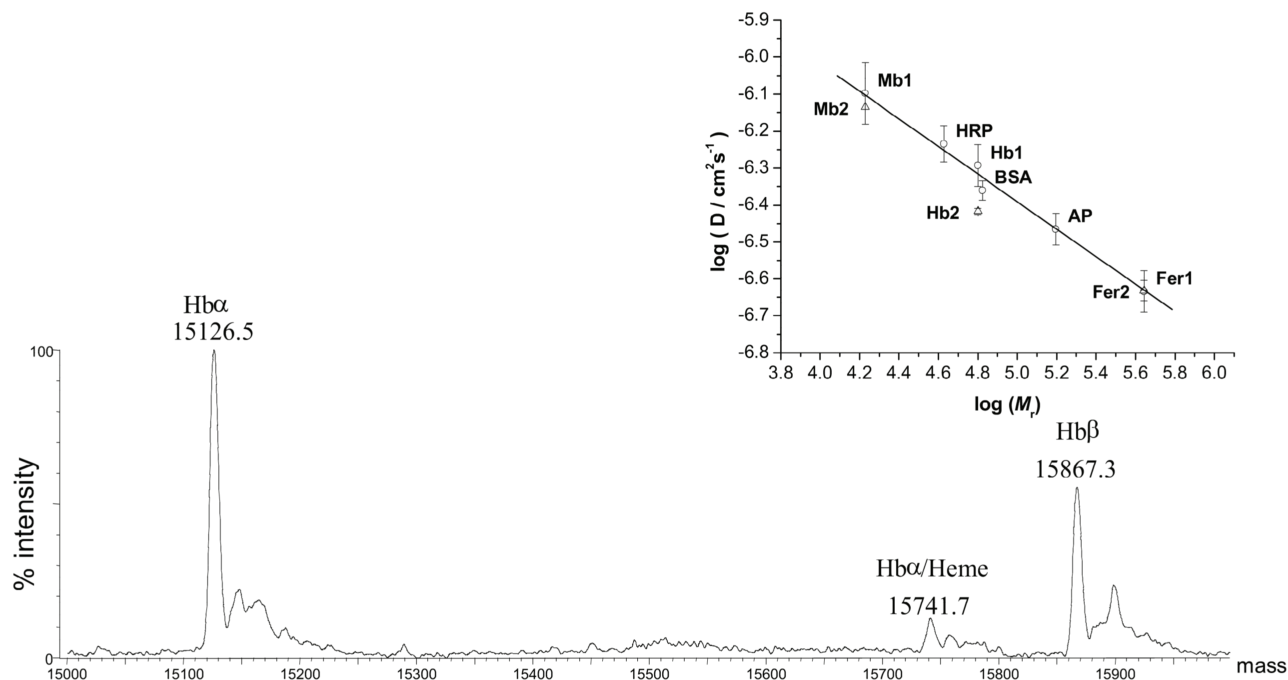

Figure 2. HF5-ESI/TOFMS of human hemoglobin (Hb): molar mass spectrum. F_{in} = 0.70 mL/min; F_{rad} = 0.38 mL/min. In the inset: regression plot log D vs. log M_r: (○) 30,000 M_r HF cut-off; ( ) 6000 M_r HF pore cut-off; Mb= horse heart myoglobin, BSA=bovine serum albumin, AP= calf intestine alkaline phosphatase, HRP=horseradish peroxidase, Fer=horse spleen ferritin. Adapted with permission from ref. 27, © 2005, American Chemical Society.

able to effectively fractionate serum proteins, as shown in Figure 1 [6]. Effective fractionation of HSA and other serum HAPs under native conditions allows, in perspectives, using HF5 for proteomic studies on peptides/proteins associated to HAPs.

HF5 can be online coupled to ESI/TOF MS by simply connecting the detector outlet to the ESI source. Results on model proteins have shown that during fractionation the proteins maintain their native structure, and are effectively desalted [7]. Correlation between the M_r values independently measured from ESI/TOF MS spectra and from HF5 retention time measurements can then provide significant information on the quaternary structure of the fractionated proteins. In Figure 2 (adapted from ref. 7) it is reported an example of HF5-ESI/TOF MS of human hemoglobin (Hb). The representative 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 ± 0.3, M_r = 15,867.3 ± 0.5, respectively), and of the α-heme complex (M_r = 15,741.5±0.7). This corresponds to the spectrum of native Hb, and the M_r value obtained from HF5 retention of Hb at pH 7.0 (data point Hb1 of the regression plot inset in Figure 6) corresponds to the M_r value of the tetramer. Since similar mass spectrum was obtained also at pH 8.2, the difference in retention observed by increasing pH (data point Hb2 of the regression plot) could be ascribed exclusively to the conformational changes that are known to occur in Hb with increasing pH.

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