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Flow field-flow fractionation: A pre-analytical method for proteomics

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ABSTRACT

Proteome analysis requires a comprehensive approach including high-performance separation methods, mass spectrometric analysis, and bioinformatics. While recent advances in mass spectrometry (MS) have led to remarkable improvements in the ability to characterize complex mixtures of biomolecules in proteomics, a proper pre-MS separation step of proteins/peptides is still required. The need of high-performance separation and/or isolation/purification techniques of proteins is increasing, due to the importance of proteins expressed at extremely low levels in proteome samples. In this review, flow field-flow fractionation (F4) is introduced as a complementary pre-analytical separation method for protein separation/isolation, which can be effectively utilized for proteomic research. F4 is a set of elution-based techniques that are capable of separating macromolecules by differences in diffusion coefficient and, therefore, in hydrodynamic size. F4 provides protein separation without surface interaction of the analyte with packing or gel media. Separation is carried out in an open channel structure by a flow stream of a mobile phase of any composition, and it is solely based on the interaction of the analyte with a perpendicularly-applied, secondary flow of the fluid. Therefore, biological analytes such as proteins can be kept under a bio-friendly environment without losing their original structural configuration. Moreover, proteins fractionated on a size/shape basis can be readily collected for further characterization or proteomic analysis by MS using, for instance, either on-line or off-line methods based on electrospray ionization (ESI) or matrix-assisted laser desorption–ionization (MALDI). This review focuses on the advantages of F4 compared to most-assessed separation/isolation techniques for proteomics, and on selected applications based on size-dependent proteome separation. New method developments based on the hyphenation of F4 with on-line or off-line MS, and with other separation methods such as capillary isoelectric focusing (CIEF) are also described.

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1. Introduction

1.1. Current proteomics needs pre-analytical methods

Mass spectrometry (MS) is rapidly maturing as a powerful method for proteomics applications. An excellent focus on this subject was recently published [1]. However, the complexity of proteomes usually exceeds the resolution capabilities of sophisticated current MS techniques either by bottom-up or...
top-down approaches. The availability of so-called “pre-analytical methods” for protein isolation/separation from complicated biological samples is required for successful MS-based approaches to current proteomics.

In clinical proteomics, many efforts have been devoted to mining this huge wealth of proteins and polypeptides present in biological fluids. MS techniques with soft ionization sources and time-of-flight (TOF) analyzers including hybrid quadrupole-TOF (Q-TOF) are most applied methods for bottom-up approaches to clinical proteomics. Several studies have shown that biomarkers can be identified on the basis of the presence/absence of multiple low molar-mass (low-M_r) serum components. However, the precise nature of all the peptides present in human serum, most of which are fragments of larger proteins, still remains largely unknown. On the other hand, few high-abundant proteins (HAPs) represent most of the protein content in biological fluids, with thousands to millions of low-abundant proteins (LAPs) that constitute a few percent 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. This still induces serious concerns on the actual applicability of clinical proteomics, as reported in a recent commentary [2].

Functional proteomics, which takes into account how native proteins interact with surrounding proteins or molecules to eventually modify the protein structure, requires very accurate measurement of the actual molar mass. Electrospray ionization (ESI) is particularly suited to top-down, MS-based identification and structural characterization of intact protein and protein complexes. In the case of complex protein samples (e.g. cell lysates), direct ESI/TOFMS shows, however, limited success, mainly because the resulting spectra are often very complicated to interpret. Rapid and efficient pre-analytical methods able to purify and simplify the sample, and to affect neither the three-dimensional structure nor the non-covalent chemistry can significantly enhance the power of ESI/TOFMS methods applied to functional proteomics.

1.2. Pre-analytical methods: “pros” and “cons”

Protein separation using one-dimensional or, most often, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is widely applied to both qualitative and quantitative approaches to proteomics, due to PAGE simplicity and ability to resolve several thousand protein spots [3–5]. Since protein separation by 2D PAGE is carried out in two orthogonal dimensions by differences of isoelectric point (pI) and molar mass (M_r) values of the proteins, separation power can be greatly increased by multiplication of the peak capacity of each technique [6]. 2D PAGE is also a relatively inexpensive method which offers unparallel ability to compare, by immediate visualization, the differences in protein expression. However, 2D PAGE presents some weak points for further MS characterization of the separated proteins. When separation is completed, proteins are in fact left in the gel matrix. This makes it difficult to retrieve protein spots in their intact conditions, and with total recovery. The latter constitutes a serious limitation for the isolation and characterization of the LAPs, even when a high-sensitive, bottom-up, MS-based proteomic approach is applied [7,8]. Finally, the entire 2D PAGE process of separation (~36 h in a typical run), spot isolation, and sample preparation for further MS analysis is time-consuming, and difficult to automate. This aspect involves elevated labor costs.

Free-flow electrophoresis (FFE) has recently gained popularity for semi-preparative scale separation of proteins based on their differences in pI. Since sample injection is made continuously through flowing streamlines, it provides unlimited throughput [9,10]. However, separation is based only on pI differences, the carrier ampholine solution is expensive and it must be removed when collected protein fractions are to be further processed by MS.

Capillary zone electrophoresis (CZE) is a highly efficient separation method, hence it has become popular also for protein separation. When coupled with MS methods, because its “nano-scale” character, CZE is considered to be among most promising techniques for “single-cell proteomics” [11]. Limitations due to low sample loadings, which may affect MS detection limits, could be partly overcome by on-line coupling CZE with nanospray MS sources. In fact, it is recognized that some technical limitations still hinder the development of on-line CZE–ESI/MS as a routine-based method. For instance, if saline buffers at relatively high ionic strength are used in CZE, they cannot be directly interfaced to ESI/MS without a proper on-line desalting device, since non-volatile salts cause serious problems in MS analysis. Moreover, issues related to possible interferences caused by the very high voltage applied in CZE to the lower voltage applied in the ion source are not, as yet, completely solved.

Reversed-phase (RP) HPLC is perhaps most established, and most technically developed separation technique. It is often used to separate protein samples before MS. It has the additional advantage to desalt the sample. However, it seldom provides enough resolution. Increase in separation performance is achieved by decreasing the diameter of the packing materials or by using narrow-bore, long columns under high or ultra-high pressure conditions (RP UPLC). Upon these pressure conditions, however, protein degradation may occur. Organic modifiers used in RP HPLC mobile phases can also induce protein denaturation, which is a limitation in functional proteomics. Multidimensional LC is sometimes preferred to increase separation performance through orthogonal separation methods. However, undesired interaction between proteins and stationary phase, which generally cause protein adsorption or entanglement affecting protein recovery and separation, have more chances to occur in multidimensional LC.

Variation in structural flexibility, stability, and morphology of the proteins reflect into changes in protein diffusion coefficient without changes in protein molar mass. Even though multidimensional LC and CZE are high-resolution methods, they are not particularly selective with respect to changes in the protein diffusion coefficient. SEC separation depends on the protein structure. SEC is then widely used as pre-analytical method for functional proteomics, and it is applied also in preparative scale. However, interaction between protein and packing material might occur, which can cause protein entanglement.
It must be finally noted that, when all the above methods are used as pre-analytical steps for MS-based proteomic approaches to biological fluids such as blood serum or urine, methods that are able to remove the HAP components before MS are still required. This is because overloading effects due to the HAP components dramatically decrease the separation performance. Surface-enhanced laser desorption/ionization (SELDI) for TOFMS allows for a separation-less, selective capture of proteins according to their structure and physicochemical properties, though leading approaches for HAP depletion are still based on immunoaffinity separation. Immunoaffinity methods however suffer from intrinsic limitations, due to dilution of the so-depleted samples, and to the possible co-depletion of species that are associated with the removed HAP components. This can seriously hinder the possibility to investigate HAP/biomarker interaction and, consequently, to recognize biomarkers that are possibly carried by the removed HAP components. This may be, for instance, a limitation also when protein microarray formats are used for biomarker discovery. Unspecific immunoabsorption can also reduce depletion specificity and, in the case of immunoaffinity chromatography, the risk of run-to-run sample carry-over is also present, which can affect accuracy and reproducibility of the obtained protein mass/charge profiles. The cost of the immunoabsorbents is also very high. Depleted sample desalting is also necessary prior to MS analysis.

The use of beads with different functionalities was reported to effectively capture and concentrate serum proteins before MS. In common with immunoaffinity methods, these methods however suffer from the potential co-depletion of those biomarkers that possibly form complexes with the HAP components. Most recently, the use of a library of combinatorial ligands coupled to beads has shown to “equalize” the protein content of biological fluids such as human blood serum and urine, reducing the concentration of the HAPs and simultaneously enhancing the concentration of the LAPs [12]. In general, however, bead-based methods do not perform an actual, time-resolved separation and collection of the different HAP and LAP components, and they may require separate sample desalting steps before MS analysis.

### 1.3. Flow field-flow fractionation

Field-flow fractionation (FFF) is, like LC, a flow-assisted separation technique. It is ideally suited to the separation of macromolecular, supramolecular and particulate analytes [13]. Both LC and FFF methods use approximately the same experimental set-up, with an FFF channel that replaces the chromatographic column. As in LC, FFF starts with the injection of a narrow sample band into a stream flowing through a thin, empty channel. The flow stream drives sample components along the channel, eventually flushing them out into a detector and/or collection device for further characterization. Nonetheless, separation is based on a totally different mechanism, to which are due the main advantages of FFF for the separation of proteins. In FFF, in fact, separation does not rely on interaction of the analyte with a stationary phase but with an externally generated field, which is applied perpendicularly to the direction of the mobile phase flow. This makes the field-flow dualism for fractionation.

The FFF separation mechanism is rather straightforward. Because of the different molar-mass size and/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. In parabolic flow conditions, the flow velocity at the channel wall is zero, and it increases to the channel center where it reaches the maximum velocity. The different analytes then are swept down the channel at different speeds, and they exit the channel at different retention times. Retention can be then expressed as [13]

\[
\frac{1}{R} = \frac{\tau_t}{t_f} = \frac{w}{D} \frac{|F| w}{6kT}
\]

where \( R \) is defined, as in LC, the retention ratio, that is the ratio between the void time \( (t_v) \) and the retention time \( (t_r) \). Thus \( t_r \) is proportional to the force \( (F) \) of the applied field. This is the fundamental expression relating retention time to the analyte properties.

Different FFF variants originate from the applied field type. In flow FFF (F4), the field consists of a second stream of mobile phase that is applied along the channel section, thus named crossflow. In F4, two flow streams are superimposed, as shown in Fig. 1. The longitudinal and crossflow streams drive the analytes toward the channel outlet and the accumulation wall, respectively. The driving force in F4 is the viscous force exerted on the analyte by the crossflow stream

\[
|F| = f|U| = 3\eta|U|d_H = kT|U|/D
\]

where \( D \) is the diffusion coefficient of the analyte, which is expressed by the Stokes-Einstein equation

\[
D = kT/\eta
\]

In Eq. (2) the term \( d_H \) is the hydrodynamic diameter of the analyte, \( \eta \) the mobile phase viscosity, and \( U \) is the crossflow velocity. Combining Eqs. (1) and (3), \( D \) can be expressed as

\[
D = \frac{t_r V_c w^2}{6 \ln V_0}
\]

where \( V_c \) is the volumetric crossflow rate, and \( V_0 \) is the channel volume. Eq. (4) states that the retention time inversely depends on the analyte diffusion coefficient, which inversely relates to the hydrodynamic diameter. In F4, retention then linearly depends on the hydrodynamic size, and it is independent of the analyte density, as expressed in the following equation.

\[
d_H = \frac{2kT V_0}{3\eta V_c w^2 / \ln V_0 t_r}
\]

The above expression indicates that F4 not only enables fractionation of the sample components, but in principle it can be utilized for concomitant size determination of the fractionated analyte.

Because of the most universal field, F4 is capable of separating almost all macromolecules and particles (e.g. from proteins to whole cells) from 1 nm to more than 50 μm in size. The lower size limit is related to the molecular weight...
cut-off of the accumulation wall, which is usually constituted of an ultrafiltration membrane (UF) able to retain the macromolecular analytes inside the channel. F4 is flexible in channel design, and we can accordingly have symmetrical F4 (SF4) [14], asymmetrical F4 (AF4) [15], and hollow-fiber (HF) F4 (HF5) [16]. The mechanism of AF4 and HF5 are described in Fig. 2a–b, respectively.

AF4 is most successful variant of F4, and few different types of AF4 systems are commercialized. As described in Fig. 3a, in an AF4 channel two machined blocks clamp together the UF membrane and the spacer in which the channel volume is removed. The accumulation block has an inset frit panel to allow for the proper distribution of the crossflow stream. The frit also provides the membrane used as accumulation wall with a rigid support. The frit then must be perfectly flat and parallel. Ceramic and stainless steel frits with 2–5 μm pores are most currently used in commercial channels. When selecting the UF membrane used at the accumulation wall, not only the pore size cut-off value but also some technical aspects must be considered, including compatibility with the mobile phase, and the most proper UF material to minimize analyte/membrane interaction, which affects sample recovery.

Over almost two decades, tubular, HF membranes employed for microdialysis applications have been utilized as cylindrical, micro-column channel for F4 [17]. The elution mechanism in HF5 follows the basic principles of F4, and it is depicted in Fig. 2b. As in AF4, the $t_r$ values in HF5 are directly proportional to the analyte $D$ value, and inversely proportional

\[ D = \frac{kT}{3\pi \eta d_H} \]

Fig. 1 – The F4 mechanism. Two analytes of different sizes are driven by the orthogonal field of viscous force $F = fU$ towards the accumulation wall. Since Brownian diffusion, of coefficient $D$, counteracts the applied field, a different steady-state condition is reached for each analyte of different $D$. Under these conditions, the analyte concentration $C(x)$ is $C_0$ at the accumulation wall, and it exponentially decreases across the channel section ($x$). The two analytes are then positioned into different velocity regions within the parabolic flow profile of the mobile phase across the channel, and they are swept down the channel at different times.

Fig. 2 – Channel design and cross-section, enlarged views of a. flat-type, trapezoidal asymmetrical F4 (AF4), and b. cylindrical type, hollow-fiber (HF) F4 (HF5).
to the fiber inner radius \((r_i)\). For analytes with sufficiently high retention, \(R\) is expressed as \([18,19]\)

\[
R = \frac{t_0}{t_r} = \frac{4D}{U r_f}
\]

where \(U\) here is the radial flow velocity at the fiber wall.

HF5 is still at its prototype stage, and HF5 channels are not as yet commercialized. We home-make the channels by sheathing a piece of HF with two pieces of 1/8” O.D. glass or Teflon tube. We have up to now employed polysulfone or polyacrylonitrile HF membranes with 10,000–30,000 kDa pore cut-off (SK Chemicals, Seoul, Korea; Chemicore Inc., Daejeon, Korea). 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 \([19–21]\). The most employed HF5 channel we have designed is reported in Fig. 3b.

### 1.4. Advantages of F4

Native conformational structure and intact folding are crucial parameters related to the functional efficacy of the protein, e.g. in case of antibodies and enzymes. The diffusion coefficient \((D)\) is a fundamental parameter to determine the protein size, shape, and surface features. From experimental measurements of \(D\), interaction/aggregation between proteins can be deduced. F4 shows intrinsic advantages for the analysis in a 10^5 molar-mass range of intact proteins and protein complexes under native conditions \([22–24]\). As expressed by Eqs. (4)–(5), the \(D\) and, then, the hydrodynamic diameter \((d_H)\) values can be determined from the experimental retention time \((t_r)\) values. This is the very important feature in case, for instance, of the analysis of protein drugs with conformation-dependent biological activities. The ability of F4 to not only separate intact proteins but also measure their diffusivity was first shown in 1977 \([25]\). High-speed analysis of protein mixtures in a broad-mass range, from 29 kDa (carbonic anhydrase) up to 669 kDa (thyroglobulin), was successively reported \([26]\). F4 was also used to analyze intact proteins of different origin, such as wheat proteins \([27]\) and enzyme mutants \([28]\), as well as intact, ultra-large proteins and protein complexes \([29,30]\).

Another advantage is that F4 is a bio-friendly technique. Compared to LC, there is no stationary phase in F4. As a consequence, mechanical or shear stress on the protein molecule caused by packing material, which can cause entanglement, or alter the native conformation of protein, or induce proteins to dissociate into smaller subunits, is very little (if any) inside the empty F4 channel. On the other hand, F4 can utilize almost any aqueous solution as mobile phase, while other separation techniques utilize organic solvents (RP HPLC), surfactant (electrophoretic methods including SDS or 2D PAGE), or saline buffer solutions (ion-exchange LC). These mobile phases may indeed cause proteins to lose their three-dimensional conformations, or they may unexpectedly induce dissociation of protein complexes during separation, or they can be incompatible with further MS characterization. Moreover, selectivity of F4 in terms of differences in \(D\) is particularly high. Lastly, sample contaminants of low-molar mass such as salts are not retained in F4, due to the porous channel walls.

Protein complexes might differ according to the degree of aggregation, size, charge, density, shape and biological activity. The study of protein complexes is important in functional proteomics because changes in conformation, as well as self-association or dissociation phenomena are strongly related to the biological activity. The interaction of proteins with other proteins, protein receptors, drugs or cell metabolites is also conformation-dependent. SEC represents the reference method for the separation of protein complexes. However, F4 potentially offers higher resolution, since the \(M_r\)-based selectivity of F4 is in principle larger than that of SEC.
This was shown in a comparative study on AF4 and SEC of antibodies [31]. Otherwise, it is also acknowledged that, in practice, F4 gives lower resolution in the <50 kDa range, and better resolution for M, values higher than 100 kDa [32]. In terms of resolution, F4 can be then preferred to SEC for relatively high-M, species, and applied in a broader M, range.

1.5. Broad-range, mass characterization of intact proteins

As shown in Eq. (4), determination of protein size and, then, M, is possible from protein retention times. However, the relationship of the hydrodynamic radius with the M, of proteins depends on protein conformation. Moreover, it is acknowledged that the accuracy of F4 in M, determination is not high because theory relating retention with diffusivity does not take into account non-ideality effects on retention, which can be due, for instance, to analyte/channel interaction. Multi-angle light scattering (MALS) detection gives absolute, uncorrelated size/M, values of the fractionated proteins. Through the angular dependence of the scattered light intensity on M, MALS detection allows for the absolute determination of M, of nanosized analytes, that is without referring to calibration [33]. In 1994 it was for the first time reported F4-MALS able to determine M, distributions of the fractionated macromolecules or nanoparticles [34]. By integration of the angular distribution of the scattered light intensity over the analyte M, distribution, MALS also gives information on the analyte root-mean-square (r.m.s.) radius. The r.m.s radius represents the mass-average distance of each point in the analyte molecule/particle from the center of gravity. As expressed in Eq. (5), F4 retention is in principle related to the analyte hydrodynamic size (dH), which is the diameter of a sphere with the same diffusion coefficient or viscosity of the analyte molecule/particle. As a consequence, by comparing the r.m.s. values measured by MALS to the hydrodynamic size values measured by F4, F4-MALS allows to obtain information on the analyte shape and/or on the mass distribution inside the analyte molecule/particle. It must be noted that F4 retention is independent of the analyte density. F4-MALS then results of great interest for the biophysical characterization of intact protein and protein complexes, particularly in the case of large or very-large M, species for which F4 and MALS are characterized by high M, based selectivity and sensitivity, respectively. AF4-MALS was employed to study prion protein aggregation and find correlation between size and infectivity of the prion protein particles [35]. Optimized AF4-MALS methods to study pharmaceutical protein aggregation today appears to represent one of most outstanding applications of this hyphenated technique in the pharmaceutical and clinical fields [36-37].

Profiling of lipoprotein patterns has become, for instance, one of the most popular methods to assess lipoprotein abnormalities and the associated coronary artery disease (CAD) risk. Lipoproteins are globular micelle-like particles formed by a core of hydrophobic and neutral lipids, cholesteryl ester, and triacylglycerols, surrounded by a shell of polar lipids and proteins. It is well known that low-density lipoprotein cholesterol (LDL-C) is the most significant CAD-related risk factor, while high-density lipoprotein cholesterol (HDL-C) exhibits a protective effect. Small, dense LDL particles differ from normal-sized LDL particles in terms of metabolism and atherogenicity. Standard and inexpensive methodologies for routine LDL size measurement are not as yet available. PAGE cannot give information on lipoprotein conformation. F4 was used to study lipoproteins screened in patients with CAD [38]. Miniaturized F4 devices, either in flat-channel [39] or in HF format [40], were further used to fractionate lipoprotein fractions. On-line coupling between MALS and either commercial, flat-type AF4 or prototype HF5 was compared for size and shape characterization of serum lipoproteins [41]. HF5-MALS showed successful also for the accurate estimation of the molar-mass and size values of other protein aggregates [42].

1.6. F4 in proteomics

Although F4-MALS allows for the mass/size-characterization of intact, high-molar-mass protein and protein complexes, it does not allow for protein identification. As recently reviewed in the literature [43], off-line and on-line coupling to MS has made F4 enter the field of proteomics. First example was the use of F4 as the pre-analytical step to fractionate whole bacterial cells for their further whole-cell protein profiling by MALDI/TOFMS [44]. Species desorbed from whole bacterial cells by MALDI, and detected in TOFMS spectra are intact proteins, which can be identified through proteomic database searches.

Pre-analytical applications in the field of MS-based protein analysis received further support by channel down-scaling. HF5 was the first micro-channel variant used for MS-based protein characterization. With respect to flat-type, macro-channel F4, HF5 shows unique, intrinsic features for MS coupling: a) low channel volume (in the order of 100 μL), which reduces sample dilution; b) low flow rate conditions (as low as 200 μL/min) which, in case of on-line coupling to MS, does not require high split ratios between the channel outlet and the ionization source; c) possible disposable usage, which eliminates the risk of run-to-run sample carry-over and, then, spectra contamination. Incidentally, features a) and b) can decrease the limit of detection in ESI/TOFMS of LAPs, while feature c) is also essential to reduce biohazards. Because of the HF porosity, HF5 also gives in-line sample micro-purification/desalting during the in-channel sample focusing/relaxation before elution. Though HF5 is, still, at a trial-prototype stage, it shows a relatively good potential in the separation of intact proteins in a broad molar-mass range. HF5 was then coupled to low-fragmentation ion sources such as MALDI and ESI for MS of intact proteins. The hyphenated system is depicted in Fig. 4 (adapted from [45]). A mixture of two bacteria (B. subtilis and E. coli) was fractionated through HF5, and MALDI/TOFMS analysis was performed on each separated bacterial species [46]. MALDI/TOFMS characterization demonstrated that mixed bacteria were fully separated through HF5, because each fractionated population preserved the most characteristic ion signals from ribosomal proteins of the species without the presence of characteristic signals from ribosomal proteins of the other species. In a further study, HF5 with MALDI/TOFMS and with a chemiluminescence (CL) enzyme activity assay was used to characterize an enzyme drug (uricase) [47]. Preliminary detection and identification of sample impurities...
was performed by means of conventional methods such as RPLC–ESI/Q-TOFMS, and MALDI/TOFMS with SDS PAGE and 2D PAGE. However, RP HPLC–ESI/TOFMS and MALDI/TOFMS did not allow to definitely establish whether uricase oligomers, among which the native tetramer, were actually present in the samples. Because of the non-denaturing conditions of HF5, its use with MALDI/TOFMS and a CI enzyme activity assay allowed to relate the supramolecular structure of the enzyme with its enzymatic activity. HF5 therefore showed to be effective as a separation/purification method complementary to LC when implemented in MS-based, comprehensive approaches to the characterization of functional proteins. In a recent study, HF5 was applied to untreated, whole human blood serum [48]. As shown in Fig. 5 (from [48]), HF5 can

Fig. 4 – HF5–TOFMS system set-up. Reprinted with permission from [45], © J. Wiley & Sons, 2006.

![Fig. 4 – HF5–TOFMS system set-up.](image)

Fig. 5 – Pre-MALDI/TOFMS fractionation of human blood whole serum by HF5; 1:5 v/v diluted in the mobile phase (NH₄Ac 5 mM). (a) HF5 fractogram and fractions collected for SDS PAGE. (b) SDS PAGE of the collected fractions. HF membrane: nominal cut-off = 30000 Mₐ, nominal inner radius = 0.040 cm (referred to dried conditions), length = 24 cm. Radial flow rate ($V_{rad}$) = 0.4 mL/min, longitudinal, outlet flow rate ($V_{out}$) = 0.3 mL/min. Reprinted with permission from [48], © Elsevier Publishers.

![Fig. 5 – Pre-MALDI/TOFMS fractionation of human blood whole serum by HF5.](image)
significantly fractionate serum proteins. Effective fractionation of albumin and other serum HAPs under native conditions may allow, in perspectives, to use HFS for proteomic studies on peptides/proteins associated to HAPs (e.g. in albuminomics). In fact, current bead-based methods for HAP depletion may suffer of poor specificity and recovery, which affect the ability to identify HAP-associated proteins. As a preliminary study, sera were therefore spiked with low amounts of an artificial mixture of relatively low-Mr proteins and peptides to assess the ability of HFS to possibly recover free or HAP-associated LAPs by means of a hybrid fractionation/microfiltration mechanism.

HFS was on-line coupled to ESI/TOFMS for the characterization of intact proteins [49]. Spectra confirmed that proteins maintained their native structure, and were on-line desalted during fractionation. The possible correlation between the Mr values independently measured by ESI/TOFMS spectra and from HFS retention time measurements can produce significant information on the quaternary structure of the fractionated proteins. For instance, in Fig. 6 (adapted from [49]) the on-line HFS-ESI/TOFMS spectrum of human hemoglobin (Hb) is reported, which shows the presence of three species with Mr values corresponding to the Mr values of the α and β subunits (Mr = 15,126.5 ± 0.3, Mr = 15,867.3 ± 0.5, respectively), and of the α-heme complex (Mr = 15,741.5 ± 0.7). This corresponds to the spectrum of native Hb, and the Mr value obtained from HFS retention of Hb at pH 7.0 (data point Hb1 of the regression plot inset in Fig. 6) corresponds to the Mr value of the tetramer. Since the HFS-ESI/TOF mass spectrum in Fig. 6 was obtained also at pH 8.2, the difference in retention observed by increasing pH (data point Hb2 of the regression plot inset in Fig. 6) could be ascribed exclusively to the conformational changes that are known to occur in Hb with increasing pH.

F4 has been recently applied to the pre-analytical separation of subcellular species. This application demonstrated the potential of F4 when used as the pre-analytical step for size-dependent proteomic analysis. Size fractionation of mitochondria from rat liver was carried out using a frit-inlet (FI) AF4 channel [50]. Collected fractions of differently sized mitochondria were examined by confocal microscopy for morphological analysis, and each fraction was then lysed for 2D PAGE of the differently sized mitochondria followed by densitometric measurements to examine variation in protein spots. The different 2D PAGE maps are reported in Fig. 7. The fractions were finally characterized by shotgun analysis using nanoLC-ESI/MS-MS. Among 130 proteins that were found in the mitochondrial fractions, 105 proved to be uniquely mitochondrial. Seven among 25 proteins listed from other subcellular species were known to exist also in mitochondria. This could be explained by possible translocation or multiple localizations of proteins among different organelles, the tracing of which is one of the most challenging goals of subcellular proteomics.

1.7. Miniaturized F4 for proteomics

Increase in MS detection sensitivity is constantly sought to improve analytical methods for proteomics. With ESI/MS, this is possible by reducing the inlet flow rate. Miniaturized
separation methods are generally characterized by high efficiency under micro/nanoflow regimes. The low sample capacity is therefore compensated by a low dilution of the separated analytes. This explains the reasons for which most recent efforts have been made to improve F4 miniaturization [51–53]. These studies led to reduce the limit of detection down to 0.45 pmol of BSA at a microflow rate regime (50 μL/min).

Since microflow regimes (down to 10 μL/min for outflow rate) can provide a good opportunity to directly interface the fractionation channel with ESI/MS for top-down proteomics, miniaturized F4 systems have a greater potential in characterizing LAPs once sophisticated algorithms have been provided to identify complicated peptide/protein fragments that are randomly cleaved through miniaturized F4–ESI/TOFMS–MS.

A miniaturized FI AF4 system was utilized to fractionate on a nanometer-size scale exosomes from human neural stem cells for subcellular proteomics [54]. Exosomes are small membrane vesicles (30–100 nm) secreted by a multitude of cell types, which are involved in a wide range of physiological roles such as intercellular communication and membrane exchange between cells. The exosomes of different sizes collected after F4 in selected fractions were examined with TEM to confirm morphology, and exosomal lysates of each fraction were digested and run in nanoflow LC–ESI/MS–MS for proteome analysis. This study also demonstrated that using a miniaturized F4 system, size fractionation before shotgun, subcellular proteomics can be performed using amounts of starting material which are very small compared to what required from conventional techniques, or even from standard-scale F4 as in the above-reviewed case of size-based mitochondrial proteomics [50]. The scale-down provided applicability to subcellular proteomics in case of cells that are difficult to culture on a large scale, such as stem cells.

Miniaturized F4 systems can be coupled to orthogonal, microfluidic separation methods to obtain multidimensional systems for high-sensitivity MS analysis of complex protein samples. Recently, microbore (μ) HF5 has been hyphenated with capillary isoelectric focusing (CIEF) for the development of a 2D, rapid, gel-free separation method for nanoLC–ESI/MS-based proteome analysis [55]. Fig. 8 reports the instrumental arrangement of the CIEF–μHF5 prototype. 2D protein separation was effectively carried out by using, as first dimension, CIEF in a Teflon capillary to exploit differences in the protein isoelectric points (pI), followed by sequential injection of pH-based fractions to the μHF5 (second dimension) to separate the proteins based on differences in the diffusion coefficient. As 2D PAGE, CIEF–μHF5 separates intact proteins based on the differences in pI and Mr but it does not utilize gel. CIEF–μHF5 then maintains the advantage of μHF5 to carry on separation in empty ducts, which is key point not to degrade proteins nor to reduce their recovery. CIEF–μHF5 provides, during second-dimension μHF5, the additional advantage of removing through the HF wall the ampholyte solution used for first-dimension CIEF. Some results of the so-obtained 2D separation with protein standard mixtures are shown in Fig. 9 (from [55]). It was noted that molecular conformation of yeast alcohol dehydrogenase (YADH, 150 kDa) (peak no. 5, Fig. 9a) was not altered during CIEF–μHF5. This was confirmed by comparison of the YADH retention times observed with or without CIEF: the values were similar. However, the same protein appeared in the 2D SDS PAGE map (Fig. 9b) as a spot between 36.5 and 55 kDa, according to the marker protein spots present in the right side of the gel map. This finding suggested that YADH subunits were likely dissociated due to SDS denaturation. CIEF–μHF5 was then applied to a human...
urinary proteome sample. The collected fractions from CIEF–μHF5 were tryptically digested, and further examined by nanoLC–ESI/MS–MS. Identification of 114 proteins was possible, including well-known biomarkers of acute-phase reactive proteins.

Most recently, μHF5 was employed for the Mr-based fractionation of the Corynebacterium glutamicum proteome [56]. C. glutamicum is a well-known soil bacterium, which is widely used in bio-industry since it secretes a high amount of glutamic acid. The use of μHF5 improved protein identification using a shotgun proteomic approach by nanoLC–ESI/MS on the fractions of different Mr values. With pre-analytical μHF5, ionization suppression and MS-exclusion effects from spectral congestion can be also reduced. Proteins identified in a digested mixture of C. glutamicum proteome by direct nanoLC–ESI/MS–MS were compared with those identified using pre-analytical μHF5. A total of 415 proteins were found, with 203 proteins commonly found with both the methods (with or without pre-analytical μHF5). However, pre-analytical μHF5 provided 90 more proteins that were not found by only nanoLC–ESI/TOFMS–MS.

1.8. Perspectives

The development of coupled, multidimensional methods appears to be particularly promising to make F4 evolve to a mature, pre-analytical phase for comprehensive, analytical approaches to proteomics. Such a kind of evolution could completely reveal the effectiveness of F4 for applications in which the gentleness of first-dimension fractionation represents a unique feature for maintaining the native conditions. Work in is on progress to apply AF4–MALS on serum samples for an approach to lipoproteomics. The Mr range values of the serum fractions obtained by AF4 are on-line determined by MALS, and digested fractions are further analyzed by nanoLC–ESI/MS–MS. This approach shows promising for the development of a method based on integrated techniques for the identification of proteins and protein complexes that are natively present in complex biological samples.

Possible coupling with other MS techniques shows also of great potential interest. For instance, inductively-coupled plasma (ICP) MS was on-line coupled to flat-channel F4 for the analysis of metalloproteins and related metal speciation [57].

Miniaturized F4 appears particularly suited to the development of multidimensional devices to be on-line coupled with MS. We however believe that quite a few technical developments are still necessary to evolve miniaturized F4 technology from prototypes to commercialized, routine techniques for the booming field of proteomics. To this end, optimized channel design and system engineering to properly assess microflow conditions, as well as system operation automation such as flow pattern commutation and flow programming should be accomplished.

Fig. 8 – Schematics of on-line CIEF–μHF5 for 2D, non-gel based protein separation.

Fig. 9 – (a) μHF5 fractograms without CIEF and after CIEF: (1) horse myoglobin (16.9 kDa, pl 7.2), (2) trypsinogen (24 kDa, pl 9.3), (3) carbonic anhydrase (29 kDa, pl 5.85), (4) BSA (66 kDa, pl 4.8), and (5) YADH (yeast alcohol dehydrogenase, 150 kDa, pl 6.23). Flow rates: inlet flow=0.6 mL/min; outlet flow=60 µL/min. After CIEF, protein bands at four consecutive pH intervals (pH 3–5, 5–6, 6–8, and 8–10) were injected into μHF5. Flow rates: outlet flow=60 µL/min; radial flow=540 µL/min. (b) 2D PAGE for five proteins: YADH appears as dissociated subunits. Reprinted with permission from [55], © American Chemical Society, 2006.
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