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Review

Field-flow fractionation in bioanalysis: A review of recent trends

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ABSTRACT

Field-flow fractionation (FFF) is a mature technique in bioanalysis, and the number of applications to proteins and protein complexes, viruses, derivatized nano- and micronsized beads, sub-cellular units, and whole cell separation is constantly increasing. This can be ascribed to the non-invasivity of FFF when directly applied to biosamples. FFF 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 analytes with a perpendicularly applied field. For these reasons, fractionation is developed without surface interaction of the analyte with packing or gel media and without using degrading mobile phases. The fractionation device can be also easily sterilized, and analytes can be maintained under a bio-friendly environment. This allows to maintain native conditions of the sample in solution.

In this review, FFF principles are briefly described, and some pioneering developments and applications in the bioanalytical field are tabled before detailed report of most recent FFF applications obtained also with the hyphenation of FFF with highly specific, sensitive characterization methods. Special focus is finally given to the emerging use of FFF as a pre-analytical step for mass-based identification and characterization of proteins and protein complexes in proteomics.

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1. Introduction

The explosive request of methods for the analysis of complex biological samples has involved a continuous development of improved separation techniques with a wide range of applications, adequate resolution, and versatility. Liquid chromatographic (LC) and capillary electromigration techniques are the separation methods most widely used and applied to a large variety of samples of biological interest. Field-flow fractionation (FFF) is also emerging in this field for its unique peculiarity to separate macromolecular, supramolecular and particulate analytes in a very broad molar mass range and under mild instrumental conditions [1]. The FFF principle 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. Under these conditions it is possible, for instance, to separate directly in a given biological fluid functional proteins like enzymes while keeping their activity, living cells, non-covalent aggregates or adducts. This opens a large number of analytical opportunities in functional proteomics, protein drug characterization, cell sorting and in biosciences in general.

The FFF mechanism and the elution modes have been exhaustively described in previous literatures [2,3]. Briefly, in FFF the separation is achieved within a capillary, empty channel in which a laminar flow of mobile phase sweeps sample components down the channel. The field is applied perpendicularly to the parabolic flow to drive the analytes into different laminar flows due to differences in their size, density, and surface properties, resulting in different retention times. In the normal elution mode, retention times are shorter for lower molar mass/size analytes. When analyte diffusion becomes negligible, as in the case of micronsized particles, the elution order is in fact reversed. That is, larger particles are eluted more rapidly than smaller particles. The elution mode is called steric/hyperlayer, and retention depends on size and other physical features of the sample particles, such as their shape, density, rigidity, and surface features. No matter the elution mode, the retention mechanism is always sufficiently "soft" to fractionate analytes in their native conformation, making FFF particularly interesting for applications in bioanalysis.

2. FFF technologies and devices

The basic configuration of most common FFF devices is based on a rectangular, flat-type channel obtained by cutting a plastic, thin foil that is sandwiched between two flat walls. Depending on the applied field, different technical implementations are required.

The use of a second flow stream as the hydrodynamic field to develop separation makes the flow field-flow fractionation (F4) technique. This is in general the most developed and applied FFF methodology, which can be found in the market as symmetrical F4 (SF4) [4] or asymmetrical F4 (AF4) [5] variants. The latter is characterized by only one permeable channel wall, which is an advantage in terms of channel simplicity and cost. AF4 uses only one pump to generate both the longitudinal and the cross-flow, and it allows for sample focusing before the elution, which is also an advantage in terms of separation efficiency. This variant has been finding the broadest application, and it is commercialized by Wyatt Technology Europe (http://www.wyatt.de/) and by Postnova Analytics (http://www.postnova.com/). The latter company also commercializes F4 technology to realize hydrodynamic (in-flow) sample relaxation (frit-inlet, FI F4 [6]) and outlet sample concentration [7] to increase sample detection. Application of a cross-flow has been found also possible using a cylindrical, porous channel. The variant, which employs a polymeric or ceramic hollow-fiber (HF) as fractionation channel (HF5) [8], shows promising features, though it is still at a prototype stage. HF5 has been showing a fractionation performance that is comparable to that of flat-type F4. It is also a micro-volume technique since the channel volume is about 10-fold lower than the volume of commercial flat-type channels [9]. The low-cost of the HF channel allows for possible disposable usage, and this is particularly relevant when used for biological samples to minimize biohazard, reduce sterility issues, and avoid run-to-run sample carry-over.

In centrifugal, sedimentation FFF (SdFFF) the channel is spooled inside a centrifuge bowl [10]. To avoid carrier liquid leakages, the SdFFF apparatus requires sealing parts spinning at high-speed, which involves some technical complexity. The SdFFF technology was implemented into an ultracentrifuge, and it was commercialized as SF^3 by DuPont. SF^3 allowed high-speed rotation and, therefore, the application of high sedimentation fields. This allowed to broad the limit of application from particles to high molar-mass bio-analytes such as nucleic acids [11–13]. However, likely because of the high investment and maintenance costs, SF^3 did not fulfill market expectations and commercialization was suspended by Dupont. A lower-intensity field, SdFFF machine is currently commercialized by Postnova Analytics.

Since early FFF developments, the application of Earth's gravity as sedimentation field (gravitational FFF; GrFFF) has been proposed [14]. Application of Earth's gravity makes GrFFF the simplest technique from a technical point of view. The channel is made of glass or plastic walls, and it can be inserted into a system for low-pressure LC or FIA [15,16]. GrFFF is still a prototype technology, whose application is limited to analyte particles that are sufficiently big and/or dense to sediment in the Earth's gravity.

Other variants using different fields such as thermal (thermal FFF; ThFFF) or electrical (electrical FFF; ElFFF) fields have been also applied to bioanalytes, with their microfluidic variants showing interesting peculiarities that are typical of microfluidic separation systems [17]. FFF-like systems using split-flow thin cells (SPLITT) for continuous, preparative-scale fractionation, of macromolecules and particles are also present in the market (from Postnova Analytics). SPLITT develops in a thin, rectangular channel where flow splitters are located at both ends of the channel. Samples are separated on a preparative scale into two size-based fractions [18,19].

3. From stand-alone to hyphenated FFF

Because of the intrinsic possibility to obtain a size/mass characterization of the analyte, pioneering applications in the bioanalytical field were in most cases based on stand-alone FFF, and oriented for both the separation and the biophysical characterization of the analytes [20]. A list of pioneering applications of FFF to bio-analytes ranging from proteins to whole cells is reported in Table 1. However, thanks to the unique peculiarity of FFF over conventional separation techniques to perform a non-invasive separation or enrichment of the analytes from complex biological systems like biological fluids or cell lysates, its integration with other analytical methodologies soon showed appealing perspectives. Hyphenation of FFF with high-sensitivity, orthogonal methods has recently shown to substantially amplify the

Table 1
Pioneering applications of FFF in the bioanalytical field.

Year	Technique	Sample	Results
	Flow FFF (F4)	Proteins	Symmetrical F4:
1977, 1999, 1999			–Determination of diffusivity and separation of intact proteins [21–23].
2003			–Molar mass determination of intact, ultra-large proteins and protein complexes in combination with light scattering [24].
			Asymmetrical F4:
2001			–Fractionation of protein mixtures [25].
2004			–Study of protein interactions [26].
			Hollow-fiber F4:
1996			–Fractionation of intact high-molar mass proteins [27].
		DNA	Symmetrical F4:
1993			–Separation of DNA at different conformation and measurement of diffusion coefficients [28].
2001			–Separation of cationic lipid–DNA complexes using online UV, multi-angle light scattering and refractive index detectors [29].
			Asymmetrical F4:
1988			–Fractionation of plasmid fragments [30].
1999			–Separation and quantification in one single analysis of tRNA in recombinant *E. coli* [31].
2003			–Determination of the protein production levels related to tRNA levels in bacterial cells [32].
		Particles	Symmetrical F4:
1996			–Accurate size determination of *E. coli* ribosomes [33].
			Asymmetrical F4:
1997, 1998			–Association of the protein production levels in recombinant *E. coli* cells to the ribosomal content [34,35].
		Viruses	Symmetrical F4:
1977			–Determination of virus diffusivity [36].
1998			–Size-characterization of the tobacco mosaic virus with multi-angle laser scattering [37].
		Cells	Hollow fiber F4:
1991, 2002, 2003			–Separation of different types of cells [38–40].
1984	Centrifugal sedimentation FFF (SdFFF)	DNA	–Fractionation of λ DNA and smaller supercoiled plasmids in their native conformations [11].
1975			–Separation and molar mass determination of the T2 phage [41].
1980		Viruses	–Fractionation of oligomeric aggregates of rod-shaped viral particles of nuclear polyedrosis virus (NPV) [42].
1981,1985			–Determination of molar mass and density of viruses [43,44].
1988		Particles	–Gentle fractionation of a wide variety of sub-cellular particles [12].
1984, 1997, 1997, 1999, 2001, 2001		Cells	–Size-based separation of human and animal living cells [13,45–48]; purification and enrichment of neuron cell culture from a cortical cell suspension [49].
2004			–Monitoring of inducted cell apoptosis in a human osteosarcoma cell line [50].
1992			–Sorting of whole yeast (*Saccharomyces cerevisiae*) cells [51].
1997, 1999, 2000, 2001			–High-resolution separation of bacterial cells from sediments, circulating blood or mouse ascitic fluid [52–55].
2002, 2002, 2003			–Coupling with flow cytometry for cell characterization [56–58].
1992, 1995	Gravitational FFF (GrFFF)	Cells	–Fractionation of human red blood cells [59,60].
1991			–Separation of living from dead microorganisms [61].
2002			–Sorting and quantification of *E. coli* cell subpopulations for vaccine productions [62].
2004, 2002, 2004			–Characterization of winemaking yeast strains [63–65].
2003, 2004			–Coupling with chemiluminescence for ultra-sensitive detection [66,67].
1989			–Development of hybrid variants combining GrFFF and adhesion chromatography for the separation of B and T lymphocytes [68].
1999, 2000, 2002			–Development of a hybrid variant dielectrophoretic/GrFFF (DEP/GrFFF) for the separation of a mixture of cancer cells from normal cells [69]; for cancer cell purging from normal T lymphocytes and from CD34C hematopoietic stem cells [70]; for the isolation of cell specimens for sensitive diagnosis of malaria infected cells [71].
1972	Electrical FFF (ElFFF)	Proteins	–Separation of proteins [72].
2004		Particles	–Micro-scale, sub-cellular particle separation for subcellular proteomics [17].
2000	Split-flow thin fractionation (SPLITT)	Proteins/cells	–Particle size distribution analysis and determination of protein diffusion [73].
1995, 1998, 2005			–Continuous fractionation of: whole human blood into proteins, platelets, red blood cells, and leukocytes, with centrifugal field [74]; human peripheral T lymphocytes, with magnetic field [75]; homogeneous cell hybridomes, with gravitational field [76].

analytical information obtained using sole FFF. Coupling with multi-angle laser scattering (MALS) or luminescence detection, flow cytometry (FC), and soft-impact mass spectrometry have resulted to be promising in many applications, from the biophysical characterization of bio-nanoparticles and of very/ultra-large proteins and protein aggregates under native conditions to pre-MS sample separation in proteomics, from the development of multi-analyte, flow-assisted immunoassays in dispersed phase to tag-less cell sorting methods. Such most recent applications are critically reviewed in the next sections.

4. Recent trends

4.1. FFF of cells

The main analytical problem when dealing with living cells is to achieve a sample clean-up and a reasonable separation of different cells from a heterogeneous cell preparation without affecting viability and physiology of the cells. First FFF applications focused on the possibility of collecting and characterizing viable cells after fractionation. Most recent applications have focused on the isolation of viable, homogeneous cell subpopulations to study the biological processes of the cells and/or for the use of the sorted cells for clinical and therapeutic applications. Among cellular phenomena, apoptosis induction and differentiation process represent two relevant pathways to study. SdFFF was used to monitor specific biophysical modifications occurred during chemical induction of cellular apoptosis or differentiation on polyvalent human erythroleukemia cell line [77]. The authors demonstrated a correlation between elution profile changes and biophysical modifications of the cells. SdFFF was also proposed as a method to better understand the differentiation process. Megakaryocytic differentiated cells were sorted from a human erythroleukemia cell line after induction with diosgenin, and effective enrichment of cells after fractionation was shown [78]. When pre-apoptotic cells were collected from an in vitro model constituted by a human osteosarcoma cell line and diosgenin, SdFFF showed enhanced specificity and sensitivity with respect to classical detection assays for apoptosis [79]. Based on these studies, a proprietary SdFFF-based technology to separate living human cells from biological fluids was developed [80].

Due to low cost of the instrumentation, operation and method maintenance, and of personnel training, GrFFF is particularly suited to its integration into cell characterization procedures. A proprietary technology based on GrFFF and fluorescent detection was developed to determine viability of commercial yeast strain cells [81]. GrFFF-based methods have most recently shown to be interesting for cellular applications. Neoplastic cell purging from an heterogeneous mixture of human living lymphocytes constituted of neoplastic B cells from a Burkitt lymphoma cell line and of healthy T and B lymphocytes from blood samples has been recently described [82]. DEP/GrFFF was developed to a proprietary technology for preparing smears for cythopatology or other cellular analysis [83].

Other FFF techniques have also showed promising. Separation of *Staphylococcus epidermidis* and *Rhodococcus erythropolis*, two bacteria with different morphological properties, was obtained by miniaturized ThFFF, which employs a thermal-conductivity gradient as applied field, based on the different thermal diffusion properties of the cells [84,85]. FFF using a resonant acoustic field normal to the flow direction was also applied for fast, continuous and high purity cell separation [86,87].

4.1.1. Stem cell sorting

An important cell separation challenge of booming interest is the non-invasive and fast isolation of human stem cells from clinical specimens for further applications, such as gene expression studies, cultivation, and tissue and organ regeneration. Typical cell sorting/enrichment methods are currently based on flow-assisted cell sorting (FACS) or magnet-assisted cell sorting (MACS) methods through the use of immunomarkers. However, their application to stem cells present some limits. Firstly, specific markers for pluripotent/multipotent stem cells, which do not have clearly recognizable functions, are not as yet available. Moreover, the presence of surface markers is not an evidence that stem cells are in their primitive and physiological state. Secondly, any cell labeling might interfere with the differentiation process of stem cells or affect their in vivo expansion. It is therefore highly suited that cells be unlabeled and minimally manipulated. Finally, FACS may suffer of a relatively low cell recovery. FFF is able to sort unlabeled cells based on very small differences in their biophysical properties such as size, shape, density, rigidity, and subcellular ultrastructure. It can be therefore applied to stem cell isolation/sorting, provided that stem cells differ from other cells and/or display a distribution in their biophysical characteristics. Moreover, under optimized conditions, the loss of cellular material during FFF is very little.

First application of FFF to stem cells was reported in 1996, when GrFFF was applied to micro-scale preparation of stem cells from mouse bone marrow [88]. A wide panel of applications to stem cell sorting was subsequently developed using SdFFF. From a cell suspension, fractionation and collection of embryonic stem cells at various stages of proliferation was obtained in a few minutes [89]. The collected cells were then used to derive transgenic mice by generation of chimeras. The effectiveness of SdFFF to provide selective, immature cell isolation without inducing cell differentiation was further shown by fast purification of an immature neural cell fraction from a human neuroblastic cell line [90]. SdFFF was subsequently applied to isolate neural stem cells from the avian olfactory epithelium [91]. From the sorted stem cells, reconstitution of a complete epithelium was possible, with the development of models to understand the mechanisms of olfactory neoneurogenesis.

Also GrFFF-derived methods have shown effective for human stem cell sorting. DEP/GrFFF recently shows able to up to 14-fold enrich in putative stem cells a cell suspension derived from enzyme-digested adipose tissue [92]. A GrFFF-derived method in which the flow/gravity-assisted fractionation occurs under non-equilibrium conditions has been technically implemented in a proprietary procedure to isolate/purify/sort human mesenchymal stem cells (MSCs) from clinical specimens of different source [93]. MSCs are thereby purged from contaminant cells, source-to-source distinguished by the different elution profiles, and differentiated in fractions having dissimilar commitment potential correlated to a different hierarchical level of stemness.

Although FFF methods allow for a gentle, tag-less, and high-recovery cell sorting, it must be however noted that development of high-production, FFF-based sorting methods should still require an FFF process scale-up. In fact, current FFF methods are typically able to sort as low as 10^6 cells per run. To make FFF-based sorting methods be effectively implemented in clinical, routine applications to stem cells, we then believe that future technical developments should focus on multi-channel, multi-run and automated procedures.

4.2. FFF-based immunoassays

The development of new, fast, easy, and potentially multi-analyte formats for immunoassays still represents one of most active research fields in analytical biochemistry. An F4-CL-based, solid-phase, competitive immunoassay format, in which micrometer-sized beads coated with the capture antibody are used as a solid phase, and an analyte-conjugate is used as a tracer has been reported [94]. This method is described in Fig. 1 (adapted from [20]). It offers many advantages such as fast kinetics of the immunological reaction, and possible develop-

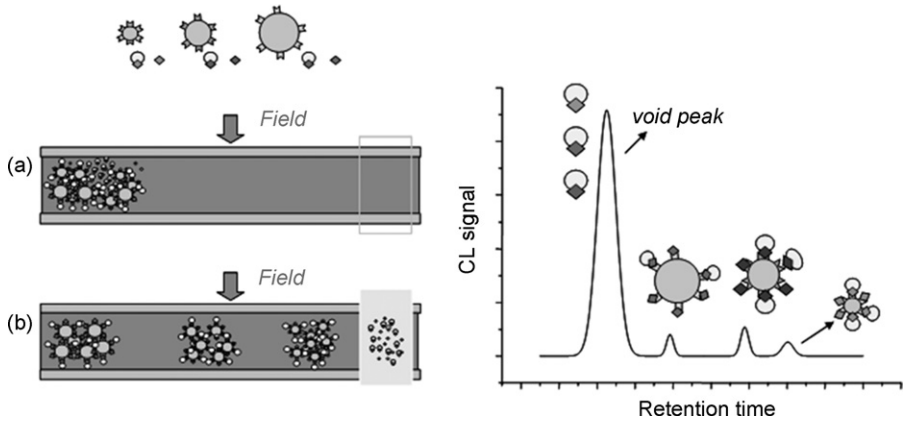

Fig. 1. The concept of FFF-based, multi-analyte immunoassay in dispersed phase. The method foresees: (a) sample injection and incubation; (b) separation of the free tracers from the bound tracers, and separation of the different tracers bound to analytes of different size; (c) quantification from peak-area. Reprinted with permission from [20], ©J. Wiley & Sons, 2006.

ment of multi-analyte FFF-based immunoassays by using beads of different sizes, each coated with the specific antibody for each analyte [95]. The performance of a suspension array where size-based separation of protein-conjugated microspheres and immuno-complexes was performed by F4 and detected by FC, was recently described [96]. The sample throughput of the suspension array was increased several times by using particles of different sizes. A side, though not trivial advantage of using F4 in bead-based immunoassay lies in the filtration through the accumulation wall membrane of low-M_r chemical species, which decreases the CL background.

GrFFF has been implemented into an innovative, non-competitive CL enzyme immunoassay for the detection of intact pathogenic bacteria in biological samples [97]. A horseradish peroxidase (HRP)-labeled monoclonal antibody is added to the sample containing the bacteria, and the mixture is immediately injected into the GrFFF channel. To make the immunological reaction take place, it follows an *in situ* incubation during the stop-flow time period before fractionation. The free and bacterium-bound antibody fractions are finally separated by GrFFF. This multi-analyte, flow-assisted immunoassay method is characterized by the use of a single antibody, and by short analysis time.

The small capillary size of the FFF channel allows to minimize diffusion and the antigen–antibody reaction occurs in few minutes rather than in hours as in the case of conventional microtiter formats. This allows for real-time format assays.

4.3. FFF of bio-nanoparticles

Science and applications of nanotechnology are developing at a very rapid pace. It is recognized that the biophysical characteristics of nanoparticles (NPs) can affect disposition in the body in ways that differ from molecular forms of same material types. Integration of information with respect to given NP material characteristics (for example size, shape, surface features) could be then of particular benefit in the field of bio-NP applications. However, it is also recognized that the development of appropriate analytical methods for nanosized bio-materials still requires substantial effort. Strengths and limitations of these methods may vary in ways relevant to evaluating characteristics such as particle size, size distribution, surface charge, surface properties, and particle interactions (such as aggregation) that may be relevant to dose, stability, or other characteristics that are significant to biological interaction or product quality. There is therefore an ultimate need to develop comprehensive analytical methods for the analysis and biophysical characterization of NPs for biological and pharmaceutical applications. In this view, FFF represents a separation method with unparallel performance.

4.3.1. NPs for drug vectorization

The particle size distribution (PSD) of nanosized, drug carrier systems is, for instance, of great influence on drug efficacy. SdFFF was used to study PSD changes of perfluorocarbon (PFC) emulsion droplets in *ex vivo* whole blood samples because of size-dependent removal of circulating NPs by monocytes and tissue-resident macrophages of the reticuloendothelial system [98]. In combination with uncorrelated mass/size characterization techniques such as MALS detection, accurate biophysical information on bio-NPs can be obtained through FFF. The PSD of drug-loaded and unloaded gelatin NPs were determined by AF4-MALS [99]. AF4-MALS was also applied to poly(d,l-lactide-co-glycolide) nanosupensions as intravenous NP systems to determine their PSD and maximum particle size, which are parameters of utmost importance for parenteral administration of the nanosuspension [100]. AF4 was recently used to determine the PSD of drug-loaded core/shell nanoparticles which have a lipid core of lecithin and a polymeric shell of a Pluronic [101]. The method provided accurate size analysis of the drug-loaded NPs without interference by the coreless micelles.

4.3.2. Virus-like NPs

AF4-MALS shows to be a powerful method also for the analysis of viruses and virus-like particles (VLPs). Despite a relative lack of published methods that still limits a widespread knowledge, it becomes acknowledged that the number of proprietary applications of this method to quality control of virus-derived vaccine productions is increasing. A method using AF4-MALS for the analysis of VLPs for new vaccine products was however recently described, and compared with dynamic light scattering (DLS) and transmission electron microscopy (TEM) methods [102]. It was therein concluded that AF4 did not induce significant aggregation, and provided accurate PSD information. AF4 combined with PDA, fluorescence, MALS, QELS, and RI detection was also employed for the development of a gene-delivery vehicle based on VLPs [103]. Molar mass, root mean square and hydrodynamic radius, composition, and purity of such bio-NPs were determined from a single analysis. AF4-MALS in combination with QELS was also applied to determine the quaternary size distribution of polyomavirus protein aggregates that are precursors of self-assembled VLPs [104].

4.3.3. Lipid aggregates and lipoproteins

Lipid NPs and liposomes are of great interest for pharmaceutical and biotechnological applications. Using SdFFF prior LS-based

sizing, the composition and stability of fluorocarbon emulsions added with triglycerides were studied [105]. SdFFF showed able to reveal two distinct populations of emulsion droplets, the presence of which was not observed via direct LS techniques due to the large scattering intensity of the triglyceride droplets. Other nanostructured lipid carriers (NLC), which were composed of oily droplets which solubilize the drug and which are embedded in a solid lipid matrix, were characterized by a comprehensive approach using SF4-MALS in combination with QELS, laser diffraction (LD), and cryo TEM [106]. The size distribution of PEG-stabilized lipid aggregates, a promising new class of model membranes, was also determined by AF4 and QELS [107].

AF4 was used in a few different studies also to characterize liposomes. The stability of zwitterionic phosphatidylcholine vesicles was investigated in the presence of chemical modifiers by monitoring changes in the liposome PSD [108]. Morphological changes in the structure of actin-containing liposomes were studied using AF4-MALS [109], and the stability of liposome-encapsulated hemoglobin (LEHb) dispersions was investigated by comparing AF4-MALS-based PSD to a theoretical model for the liposome size distribution [110]. AF4-MALS was also used to study shape, size distribution, and encapsulation efficiency of actin-containing LEHb dispersions [111]. Most recently, self-assembled liposomes were characterized using AF4 combined with MALS and QELS [112].

Lipoproteins are micelle-like, lipidic nanoparticles that are responsible of lipid transport in blood. Not only cholesterol and triglyceride levels in lipoproteins, but also lipoprotein size and shape distribution are correlated with a risk of coronary artery disease (CAD). Since early times F4 has showed effective for lipoprotein characterization [113]. Prototype systems like HF5 or miniaturized AF4 have been respectively applied for determining the reduction in Stokes' size of low density lipoprotein (LDL) particles present in blood plasma samples obtained from CAD patients with respect to healthy donors [114], and for PSD analysis of standard high-density lipoprotein (HDL), LDL, and very low-density lipoprotein (VLDL) particles [115]. Most recently, AF4 was used to determine size of spherical and discoidal HDL particles, and of small, unilamellar lipid vesicles to investigate the transfer mechanism of phospholipid-rich surface components from postlipolytic chylomicrons and VLDL to HDL particles [116]. AF4-MALS and HF5-MALS have finally proved able to profile human blood lipoproteins, and to shape-characterize the LDL class [117]. In particular, HF5-MALS has showed a performance comparable to that obtained by commercial AF4-MALS, as described in Fig. 2 (adapted from [117]).

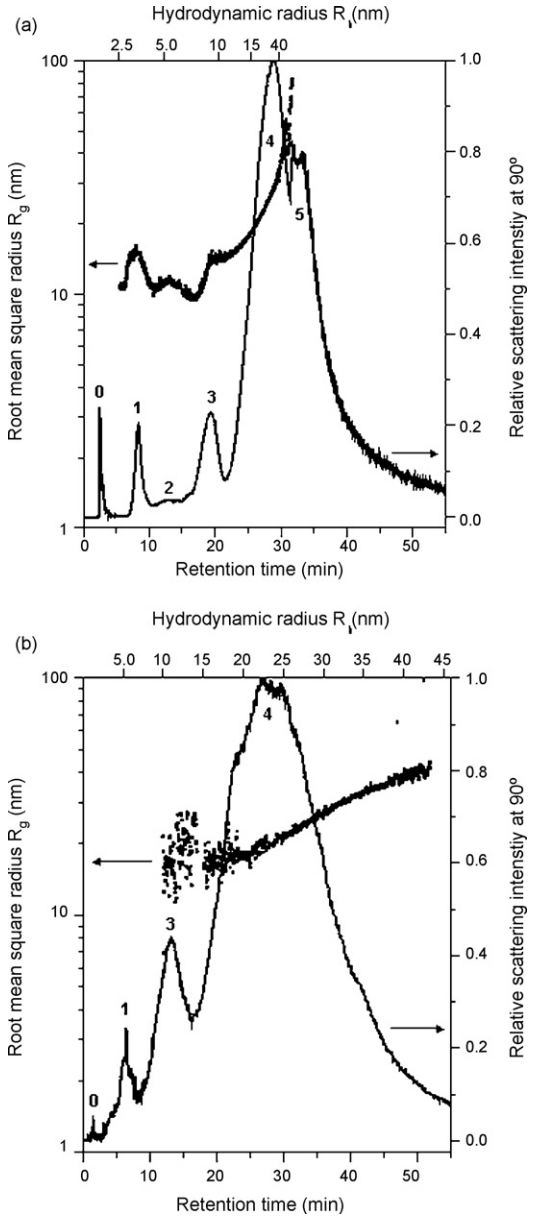

Fig. 2. (a) AF4-MALS and (b) HF5-MALS of the same serum sample (total cholesterol = 195 mg/dL, TG = 473 mg/dL). Hydrodynamic radius (Rh) calculated from F4 retention. (0) Void peak; (1) HDL + HSA, (2) IgG, (3) LDL, (4) VLDL, and (5) end of field. Reprinted with permission from [117], ©Walter de Gruyter GmbH & Co. KG.

4.4. FFF of biopolymers

Characterization of biopolymers, from polysaccharides to proteins, is one of main challenges in the eve of biotechnology. Analytical methods should not only give accurate information on the molecule structure, but also to preserve and investigate its native conformation and supra-molecular, non-covalent interactions. Few separation methods are however sufficiently gentle to fulfill these requirements. Over more than a decade, the effective application of FFF to proteins and polysaccharides has been reported [118], and the year rate of scientific publications on this topic is constantly increasing. This is because FFF techniques present intrinsic advantages for the analysis of high and ultra-high molecular weight biopolymers in native conditions. Since there is no stationary phase inside the channel, mechanical or shear stress on the analyte molecules caused by packing material, which can induce entanglement, or alter the native conformation, is very little (if any). Moreover, FFF can use almost any aqueous solution as mobile phase, while other separation techniques utilize organic solvents (RP HPLC), surfactants (electrophoretic methods including SDS or 2D PAGE), or saline buffer solutions (ion-exchange LC). These mobile phases may indeed cause biopolymers to lose their three-dimensional conformation, or to induce dissociation of non-covalent complexes during separation. Although most FFF techniques have been applied to biopolymers, today's applications are mainly focused on F4, which actually shows additional advantages. Firstly, the hydrodynamic size and molar mass of a biopolymer can be obtained from retention time. This was shown, for instance, in the case of a biosurfactant isolated from a cultivation of *Pseudomonas* sp. G11 [119]. Secondly, because of the porous accumulation wall, the macromolecular bioanalytes are purged from low-M_r components present in the sample. Finally, when combined with MALS detection, F4 shows its best for biopolymer characterization because an uncorrelated, absolute mass/size characterization can be thereby obtained [120].

4.4.1. Polysaccharides

AF4-MALS showed to be a powerful tool for characterizing polysaccharides. Programmed cross-flow in AF4-MALS was applied to determine size and molar mass distribution of ethylhydroxyethyl cellulose [121]. More recently, programmed AF4-MALS was studied to find the most-suitable flow conditions to molar mass distribution analysis of high-M_r polysaccharides [122]. With pullulan standards in the 5×10^3–10^6 molar mass range, exponential field decay was found to give the most uniform M_r-based selectivity across the fractogram. Various oxidized mono/di/tri/poly saccharides were studied as potential hemoglobin (Hb) cross-linkers. A synthetic approach was used to synthesize these carbohydrate–hemoglobin conjugates, and AF4-MALS was used to measure the absolute M_r distribution of these PolyHb dispersions [123]. Polysaccharides characterized by AF4-MALS also include alginate [124], electron-irradiated scleroglucans [125], alpha-carrageenan [126], water-soluble, non-structural polysaccharides from plants [127], and exopolysaccharides from microorganisms [128].

The applications of amylose and amylopectin, the polysaccharides obtained from the dissolution of starch granules, span from fermentation industry to biotechnology. Most technical properties of these polysaccharides are related to their size, molar mass and, in the case of amylopectin, branching degree. Since the molar mass range of amylose and amlylopectin is extremely wide, PSD analysis of these samples is particularly challenging. AF4-MALS proved to give accurate size and M_r characterization of starch derivatives. Providing an independent determination of hydrodynamic radius, gyration radius, and M_r, the polymer conformation and branching degree can be thereby obtained [129]. In recent studies, AF4-MALS was also applied to cationic starch derivatives [130,131], and to hydrophobically modified starch [132,133]. Amylose–polystyrene block copolymers were studied in organic solvent [134], and the molecular weight of amylopectin in extruded waxy maize starch samples was determined [135].

FI AF4 operating under field-programmed conditions was coupled to MALS for the size characterization of ultrahigh-M_r sodium hyaluronate (NaHA), which is important in pharmaceutical applications [136]. Hyaluronic acid (HA) is an ultrahigh-M_r polysaccharide that is found in body tissues, synovial fluid, vitreous humor, and umbilical cord. FI AF4-MALS was recently utilized for the size characterization of HA and for determining variation in molar mass distribution (MMD) and conformation of HA samples in aqueous solution when subjected to biodegradation processes such as irradiation of gamma rays [137], ultrasonication, and enzymatic hydrolysis [138].

4.4.2. Proteins

Most-developed FFF applications to biopolymers have been targeted to proteins. Although FFF using a magnetic field [139], and SdFFF [140] have been recently applied also to protein characterization, F4 (either SF4, AF4, or HF5) has been most-employed FFF techniques for protein analysis. This is because the diffusion coefficient (D) is a fundamental parameter to evaluate for the determination of protein size and shape, which reflect possible changes in the native structure of the proteins and, therefore, in their functional efficacy, as in case of antibodies or enzymes.

A series of papers have been published on F4 of plant proteins. F4 was used to investigate the subcellular location of starch-related enzymes in *Arabidopsis* mutants defective in starch degradation [141]. AF4-MALS was used to determine the MMD of wheat proteins [142], and efficient separation and size characterization of monomeric and polymeric wheat proteins was achieved in a single run. The MMD of glutenin from Australian wheat was also studied by SF4 [143], and AF4 was applied to the evaluation of dissolution methods for unreduced glutenin [144]. The molar mass of polymeric glutenins was determined by AF4-MALS as a quality parameter to assess stabiliy of bread-making quality of wheat flours [145], and for MMD analysis of glutens extracted from flours of different wheat varieties having varying baking quality [146].

The biophysical characterization of large protein complexes or protein aggregates is one of most interesting applications of F4-MALS [120,147]. AF4-MALS was employed to study prion protein aggregation and thereby find correlation between size and infectivity of the prion particles [148]. AF4-MALS not only gives information on the native aggregation of proteins, but it may also be a valuable support in monitoring biotechological processes for recombinant protein production and refolding. In a recent study, AF4-MALS was applied to the size analysis of green fluorescent protein inclusion bodies (GFPIBs) that were prepared under various culture conditions to determine the effect of culture parameters on GFPIB size distribution [149]. For the characterization of protein aggregation, a therapeutic IgG was considered as sample case. The protein solutions were characterized by a comprehensive method including microscopy, AF4, light scattering, circular dichroism, fluorescence and fluorescence lifetime spectroscopy [150]. This combined method allowed for a reliable assessment of protein self-association and aggregation phenomena. Most recently, on-line, fluorescent dye detection showed able to improve a combined method including AF4-MALS to study aggregation and structural changes of monomeric and aggregated recombinant IgG in heat-stressed formulations [151]. Calsequestrin aggregates were also analysed by AF4-MALS, supporting the hypothesis that this Ca^{2+} binding protein undergo to aggregation via interaction of dimers [152]. Despite previous reports based on SEC, AF4-MALS demonstrated that the dimer was the stable species, with very little monomer present. Glutaraldehyde-polymerized bovine hemoglobin (PolyHb) is a possible universal blood substitute. Bovine Hb was polymerized with glutaraldehyde, and AF4-MALS was used to measure the absolute M_r distribution of the PolyHb dispersions in order to evaluate the effect of varying different reaction parameters on the physical properties of PolyHb dispersions [153]. In combination with QELS, turbidity, and rheo-small angle light scattering (rheo-SALS), AF4 was recently employed to investigate the effect of pH on purified pig gastric mucine aggregation [154].

Due to the continuously increasing interest in whole protein characterization, new F4 methods have been specifically developed. A two-dimensional AF4-liquid chromatographic (AsF4-RPLC) system was presented and applied to the separation of a mixture of standard proteins [155]. The effect of heat on egg white denaturation was studied, and the unfolding of peptide bonds in the protein was found to be pronounced when the sample was heated in phosphate solution. Heat-induced aggregation of β-lactoglobulin (β-LG) in aqueous solution was studied using a HF5-MALS system in which ceramic HF were used for the fractionation channel [156].

4.5. F4 for proteomics

The outstanding capabilities of F4 for the separation of intact proteins under native conditions have recently made interesting implementation of this technique into combined methods for proteome analysis. In fact, the complexity of proteomes usually exceeds the resolution capabilities of current MS techniques either by bottom-up or top-down approaches. The availability of the so-called “pre-analytical methods” for protein isolation/separation from complex biological samples is required for successful MS-based approaches to current proteomics. In clinical proteomics, for instance, biomarkers can be identified on the basis of the presence/absence of multiple low low-M_r serum components. However, few high-abundant proteins (HAP) constitutes most of the protein content in biological fluids, with thousands to millions of low-abundant proteins (LAP) that in fact represent only 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. Functional proteomics requires very accurate measurement of the actual M_r values through top-down, MS-based identification and structural characterization of intact protein and protein complexes. The resulting spectra however 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 MS methods applied to functional proteomics. The effective use of FFF, and particularly F4 as an outstanding, pre-analytical method for MS-based proteomics has been recently reviewed [157,158]. F4 potentially offers resolution higher than in SEC for M_r values higher than 100 kDa [159], because of the higher M_r-based selectivity [160]. Low-M_r sample contaminants such as salts are not retained in F4, due to their filtration through the porous channel walls.

4.5.1. F4-MS

First example of possible use of F4 as a pre-analytical method for MS-based protein profiling was reported for whole-cell MALDI/TOFMS [161]. Pre-analytical applications in the field of MS-based protein analysis however received significant support by channel down-scaling. HF5 was the first micro-channel variant used for MS-based protein characterization. Though still at a trial-prototype stage, the intrinsic advantages of HF5 made its coupling with low-fragmentation ion sources for MS particularly effective for the analysis of intact proteins. The hyphenated system is depicted in Fig. 3 (adapted from [162]). A mixture of two bacteria was fractionated through HF5, and MALDI/TOFMS analysis was performed on each separated bacterial species [163]. When coupled with MALDI/TOFMS and with a chemiluminescence (CL) enzyme activity assay, HF5 allowed to relate the supramolecular structure of an enzyme drug (uricase) with its enzymatic activity, since RP HPLC-ESI/TOFMS and MALDI/TOFMS did not permit to establish whether uricase oligomers were actually present in the samples [164]. HF5 has been recently applied to fractionate proteins in untreated, whole human blood serum, and to possibly recover free or HAP-associated LAPs by means of a hybrid fractionation/microfiltration mechanism. [165]. As shown in Fig. 4 (from [165]), HF5 can significantly fractionate serum proteins. Effective fractionation of albumin and other serum HAPs under native conditions may allow, in perspectives, to use HF5 for proteomic studies on peptides/proteins associated to relatively abundant proteins. For

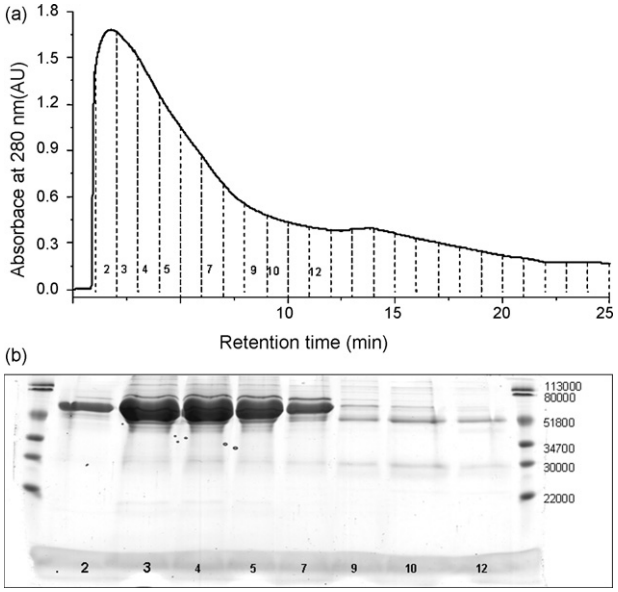

Fig. 4. Pre-MALDI/TOFMS fractionation of human blood whole serum by HF5; 1:5, v/v diluted in the mobile phase (NH_4Ac 5 mM). (a) HF5 fractogram and fractions collected for SDS–PAGE. (b) SDS–PAGE of the collected fractions. HF membrane: nominal cut-off = 30,000 M_r, nominal inner radius = 0.040 cm (referred to dried conditions), length = 24 cm. Radial flowrate (V_{rad}) = 0.4 mL min^{-1} n, longitudinal, outlet flowrate (V_{out}) = 0.3 mL min^{-1}. Reprinted with permission [165], ©Elsevier Publishers.

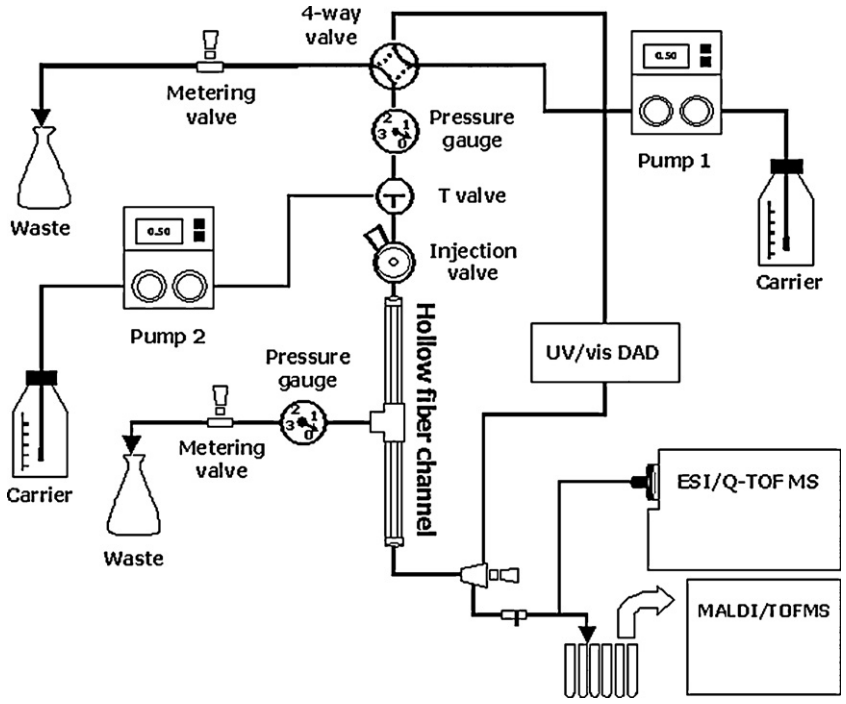

Fig. 3. HF5-TOFMS system set-up. Reprinted with permission [162], ©J. Wiley & Sons, 2006.

example, current bead-based methods for HAP depletion may suffer of poor specificity and recovery, which affect the ability to identify HAP-associated LAPs. HF5 was also online coupled to ESI/TOFMS for the characterization of intact proteins. The work showed that possible correlation between the M_r values independently measured by ESI/TOFMS spectra and from HF5 retention time measurements can produce significant information on the quaternary structure of the fractionated proteins [166].

F4 has been recently applied to the pre-analytical separation of subcellular species [167]. Size fractionation of mitochondria from rat liver was carried out using a FI AF4 channel, as shown in Fig. 5A (from [167]). Collected fractions of differently sized mitochondria were lysed for 2D PAGE, as reported in Fig. 5B (from [167]). The fractions were finally characterized by shotgun analysis using nanoLC–ESI/MS–MS. Differences in protein composition were found in differently sized mitochondria size. Among 130 proteins were found in the mitochondrial fractions, 105 unique proteins were found to be mitochondrial, and seven among 25 proteins listed from other subcellular species were known to exist also in mitochondria. Most recently, FI AF4 has been demonstrated to be a soft, preparative method to size-fractionate membrane fragments containing membrane proteins from free cytoplasmic

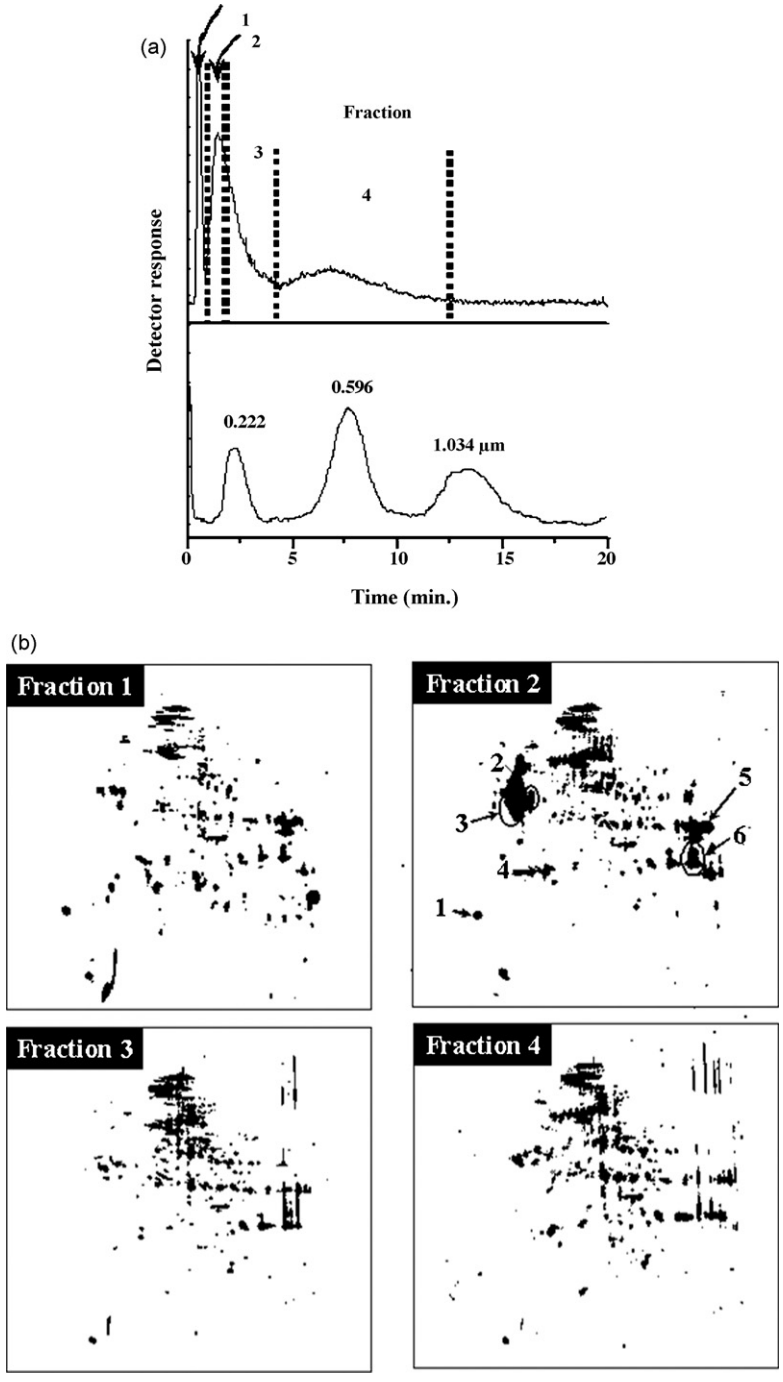

Fig. 5. (a) FI AF4 of mitochondrial extracts from rat liver: comparison with FI AF4 of PS standard latex particles. Injection flow/frit flow rate = 0.15/5.0 mL min^{-1}; outflow/cross-flow rate = 0.3/4.85 mL min^{-1}. Mobile phase for mitochondria: 0.1 M sodium phosphate. (b) SDS–PAGE gel image of mitochondrial proteins from each fraction, showing heterogeneous protein species according to their retention times. Each gel was loaded with 10 μg of mitochondrial lysate of each fraction. Reproduced by permission from [167], ©Royal Society of Chemistry, 2008.

proteins from lysated prostatic cancer cells (DU145 cell) [168]. The resulting membrane fragments were collected during the FI AF4 runs, and the membrane proteins were digested in solution for identifying proteins by nanoflow liquid chromatography/tandem mass spectrometry (nLC–ESI–MS–MS). FI AF4 was found to provide an increased yield of purified membrane proteins (172 proteins) with fewer cytoplasmatic proteins, compared to only 127 proteins found from purification process using a conventional ultracentrifugation method.

4.5.2. Miniaturized F4

Increase in MS detection sensitivity is constantly sought to improve analytical methods for proteomics. In ESI/MS, this is possible by reducing the inlet flow rate. Most recent efforts have been then focused to improve F4 miniaturization [168–170]. A miniaturized FI AF4 system was utilized to fractionate on a nanometer-size scale exosomes from human neural stem cells for subcellular proteomics [171]. Using such a miniaturized system, size fractionation before shotgun, subcellular proteomics was performed using amounts of starting material which were very small compared to what required from conventional techniques. This is key feature in case of subcellular proteomics of cells that can be hardly cultured on a large scale, such as stem cells. Microbore (μ)HF5 was recently employed for M_r-based fractionation of the *Corynebacterium glutamicum* proteome [172]. Proteins identified in a digested mixture of *C. glutamicum* proteome by direct nanoLC–ESI/MS–MS were compared with those identified using 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.

The μHF5 variant was hyphenated with capillary isoelectric focusing (CIEF) for the development of a 2D, rapid, gel-free separation method for nanoLC–ESI/MS-based proteome analysis [173]. CIEF-μHF5 showed to maintain 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. The development of coupled, multidimensional methods appears to be particularly promising to make F4 evolve to a mature, pre-analytical methodology for comprehensive, analytical approaches to proteomics.

5. Conclusions

When compared to other separation methods in the bioanalytical field, the most appealing feature of FFF lies in the fact that the fractionation process does not affect sample properties. Analytes are separated even using the same liquid media in which they are dissolved. This helps in keeping the analytes in their intact and native form, and it allows to obtain analytical information even on analytes that form week complexes with the sample components. Furthermore, in the case of living microrganisms or cells, this allows to keep them alive ND not to alter their physiology. For instance, the fractionated cells can be recovered for further cultivation or direct use. Despite these unique features, FFF has been for long time considered as the "best-kept secret" in the field of bioseparations. The most recent trends in FFF application to analytical biochemistry here reviewed would support that this is no longer the case. However, there are no doubts that FFF has not as yet "exploded" in bioanalysis, as it has been the case of LC or electrophoresis. A combination of different factors might have contributed to this slow evolution process. A first reason for such a slow evolution could be the fact that FFF is applicable to a broad range of different biosamples, but it is not as yet straightforward to decide which FFF method should be best-used for a given application. However, because of the highest flexibility, F4 nowadays is the leading and best-sold member of the family, and it will likely continue to be the most applied FFF technique. A second reason might be that FFF has been for long considered and applied as it was an "absolute" method for both separation and characterization of the bioanalytes, while well-established techniques show superior characterization capabilities. Hyphenated FFF methods can exploit the combined advantages of using FFF together with characterization techniques. We therefore expect that integration into comprehensive analytical platforms should give to FFF the most outstanding perspectives. In particular, we believe that in the near future F4-MALS and F4-MS in the field of proteomics shall represent the leading niche. Miniaturization, and particularly miniaturized F4 systems, should make increased use of FFF as a pre-analytical method for MS-based proteomics. In these regards, some technical developments, among which an optimized channel design and accurate flow controls, are still necessary to evolve miniaturized F4 technology from prototypes to commercialized techniques.

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