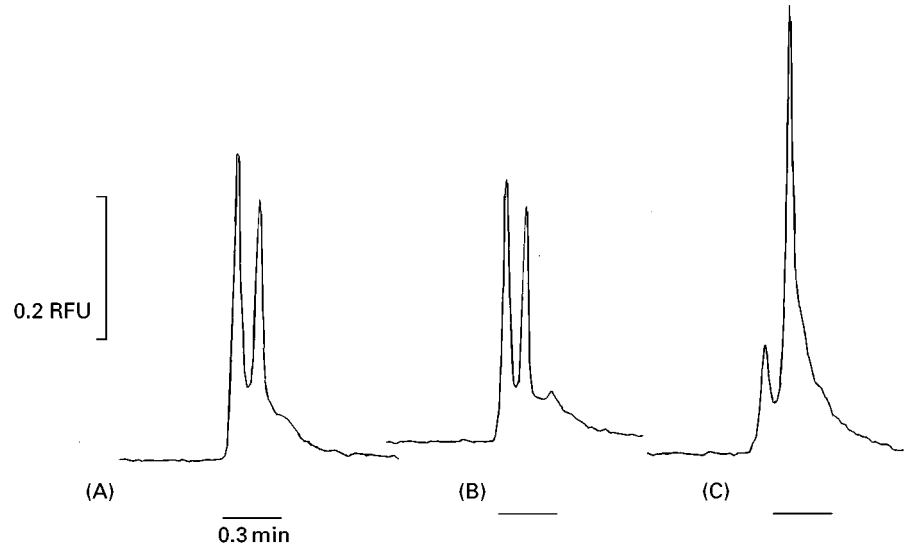

Figure 7 Representative electropherograms of (A) antibody control; (B) buffy coat from a scrapie-negative sheep; (C) buffy coat from a scrapie-positive sheep.

samples extracted from buffy coats of a normal sheep and from a buffy coat of a scrapie-infected sheep.

Concluding Remarks

The capillary electrophoresis assay described in this study is reproducible, more sensitive and faster than other analytical tests. The samples used in the capillary electrophoresis assay were obtained from brain and the lymphoid system of the animals. The sensitivity of this assay made it possible to test samples from other tissues that contain much less abnormal prion protein than brain samples. This assay has the potential to use tissues and fluids from live animals and diagnose animals prior to the onset of clinical signs of disease. Automation of this test could lead to more economical and efficient methods for testing for abnormal prion protein.

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Field Flow Fractionation

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Introduction

This review focuses on the use of field-flow fractionation (FFF) for the characterization of proteins and protein assemblies such as protein aggregates, DNA

and viruses. FFF is based on the differential transport rates of solutes in a ribbon-like channel when interacting with an applied field. The type of field may be chosen from a wide range, for example an electrical potential, sedimentation, a hydrodynamic cross-flow, a thermal gradient and so forth. A schematic of this is shown in **Figure 1**. The solute will therefore occupy a region above the sample wall, with a mean position determined by the balance between the solute's diffusion and the sample–applied field interaction. Although there exist further complications for solutes greater than ~ 0.5 μm diameter, they are not relevant given the small hydrodynamic diameter of proteins. Positioned at the outlet of the channel is a sample detector of some sort, typically a traditional high-performance liquid chromatography (HPLC) spectrophotometric detector, although a significant development has been with the application of a number of detectors providing complementary information about the sample. Such detectors include spectophotometric and refractive index types, and more recently light scattering for molecular mass, electrospray–mass spectrometry, and inductively coupled plasma, although the last two have not yet been applied to protein studies.

There are a number of advantages offered by the FFF methods over other contemporary protein analysis methods. FFF is often more rapid than analytical ultracentrifugation, and the range of fields available provide FFF with greater versatility. In comparison with gel-permeation chromatography, FFF is not impeded by a size exclusion limit, the low exposed surface area limits sample loss through adsorption on to the exposed surface, and the availability of field programming allows a wide range of materials to be analysed in a single channel. The open channel geometry usually allows FFF to characterize samples without need for pretreatment, such as filtration, and provides a very high upper limit to the protein size range. Similarly, the open channel allows the theoretical basis of FFF to provide direct access to fundamental physical constants of proteins, often without the need for calibration. Finally, both FFF and gel electrophoresis may separate a protein mixture, but sample collection is simpler in FFF.

Flow FFF

The 'universal' nature of (cross)-flow FFF (Fl-FFF) has led to its wide use for protein characterization. The free choice of carrier liquid, whether a buffer or a simulated native environment, avoids denaturing the protein. Flow FFF has two configurations, the original symmetric form and the newer asymmetric method, differing only how the field is generated in the fractionation cell and sample-loading protocols.

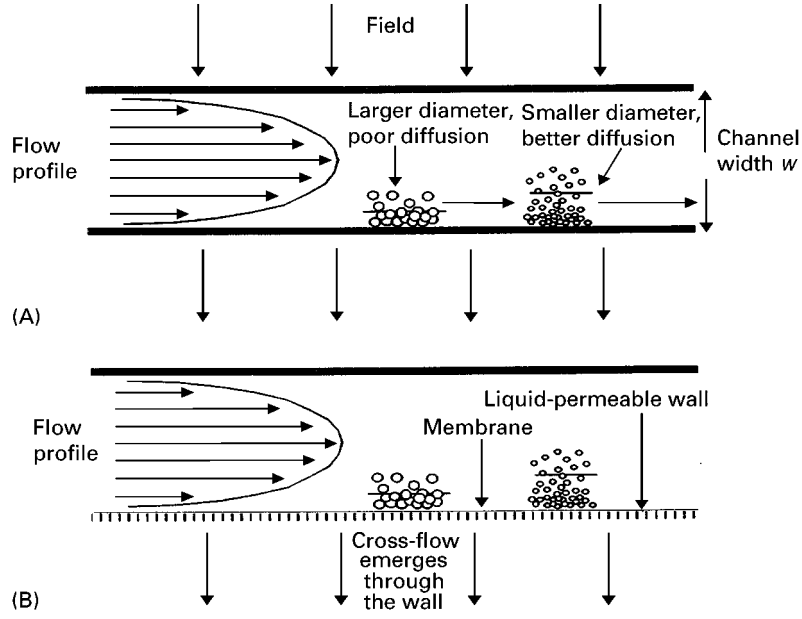

Figure 1 (A) Schematic of the mechanism of FFF separation of proteins. The smaller protein, with greater diffusivity, competes more successfully with the applied field and occupies a mean position further from the accumulation wall. Samples occupying the higher mean position is subject to more rapid flow laminae, and elutes earlier. The particle sizes represented, the channel thickness and the extent of back-diffusion are not to scale. (B) For the subtechnique flow FFF, the accumulation wall is a liquid-permeable porous material, typically ceramic. A membrane exists over the accumulation wall to prevent the samples from leaving the cell through this wall. The upper wall may or may not be porous as well, depending whether the symmetrical or asymmetrical variant is used.

Table 1 Compilation of flow FFF physicochemical data relevant for selected common proteins and with comparison to commercial polystyrene latexes

Sample	*Molecular mass* (*Da*)	*Diffusion coefficient* ($\times 10^{11}$ m^2 s^{-1}, $\times 10^{7}$ cm^2 s^{-1})
Cytochrome c (bovine heart)	13 400	11.4
Ovalbumin (chicken egg)	45 000	8.71
Bovine serum albumin	64 000	6.89
Catalase (horse liver)	221 000	4.30
Apoferritin	450 000	3.84
Urease	483 000	3.46
Ferritin	622 000	2.91
Tobacco mosaic virus	$\approx$ 40 000 000	0.46
Polystyrene latex, $\varnothing$ 0.090 μm		0.45
Polystyrene latex, $\varnothing$ 0.311 μm		0.22

In both cases, the separation is a direct function of the diffusion coefficient, where the most highly diffusive components are the least retained. A compilation of common biological samples and their diffusion coefficients are provided in **Table 1**.

Fl-FFF is capable of separating proteins with only a 15% size difference within 3–10 min. Reported results for animal proteins and biopolymers include albumins (human and bovine serum, egg), globulins (γ-globulin, haemoglobin, thyroglobulin), ferritin, apoferritin, lysozyme, casein, blood products (human and rat blood plasmas, lipoproteins) and nucleic acids. Proteins from an industrial perspective are represented by a growing body of work emerging on the characterization of proteins from flours used for bread-making purposes.

In all of the above cases, no sample treatment is needed prior to injection, such as exhaustive dialysis or filtration. This is to be expected, as the permeable membrane acts as a dialysis cell, and the open channel will not become clogged and require a filter. Since the sample is not manipulated beforehand, the presence of aggregate structures remains unaltered. **Figure 2** shows baseline resolution of a biological mixture. Protein dimers elute as satellite peaks at $\sim$1.4 retention times of the monomer, followed similarly by the higher aggregates eluting later. Most significantly, the entire separation takes place in only four minutes.

The asymmetric flow FFF variant does not inject the sample directly into the inlet line. Rather, a sample pump introduces the sample into the cell and opposing flows from both ends of the cell hydrodynamically focus the sample into a narrow band across the channel before elution. This allows for remarkably well-resolved and efficient protein separations. **Figure 3** illustrates the sensitivity of the technique. Two plasmid fragments were injected at low concentration (0.1 μg μL^{-1}) and volume (1 μL) while exhibiting both baseline resolution and elution in less than 15 min. One further advantage of the focusing method is the immobilization of the sample prior to elution. For a very dilute sample, multiple injections subject to these opposing flows produce an on-channel concentrating effect, where the protein is retained on the membrane at the focus point.

During any form of chromatography, sample dilution is inevitable. For small quantities of proteins this may challenge the limits of the detectors used. FFF offers an advantage over other methods through the ability to skim off the atmosphere of carrier liquid and greatly reduce sample dilution before detection. Sample enhancement was first mentioned in the literature in the early 1990s and now enjoys routine use in

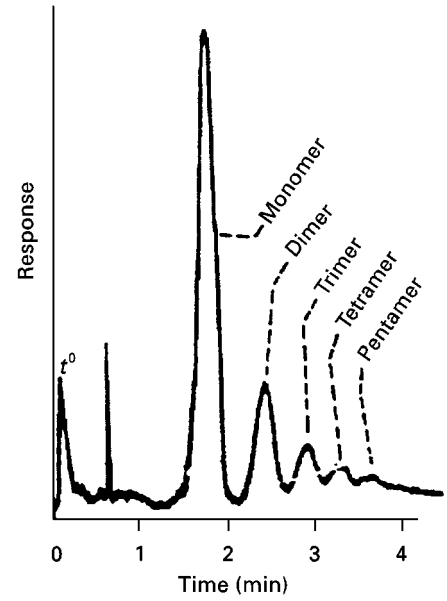

Figure 2 Separation of a monoclonal antibody from its higher clusters showing separable peaks up to pentameter aggregation. (Reproduced with permission from Giddings (1993) *Science* 260: 1456, Copyright the American Association for the Advancement of Science.)

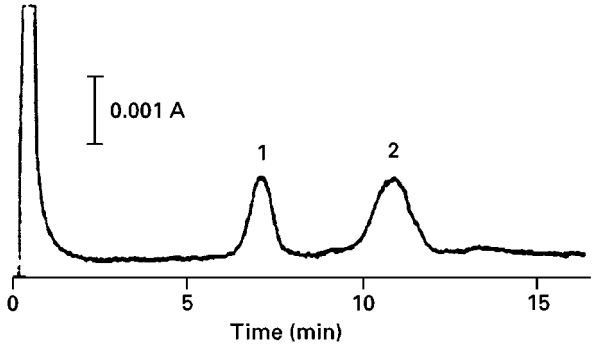

Figure 3 Separation of (1) 2390 bp and (2) 4320 bp plasmids by asymmetrical FFF. (Reproduced with permission from Litzén A and Wahlund KG (1989) *Journal of Chromatography* 476: 413 Copyright Elsevier Science BV.)

contemporary practice. Both symmetrical and asymmetrical variants have been successfully applied to proteins. Of special interest is the frit inlet–frit outlet modification. These methods in tandem enhance detectability and aid fractionation stability. The combination of frit inlet and outlet has been reported as recently as 1999, for the automation of wheat protein fractionation.

One rarely discussed drawback to the Fl-FFF method is the requirement of a membrane for sample retention. For adhesive protein samples, this demands compatibility between the sample, membrane and the carrier solution. Biopolymers can strongly adsorb on to particular membranes and at modest ionic strengths may be completely adsorbed. The simplest method to test this is to inject samples over a range of concentrations and/or volumes and ensure there is proportionality between detected signal size and the amount of sample. A partial, reversible adsorption leads to an increased retention and this would indicate that the sample is erroneously large, or induce a number of fractionation profile artefacts. Clearly the chemistry of the system, between the sample, membrane and carrier, must be known before any statements may be made.

The membrane's physical characteristics may also be significant. Firstly, membrane compressibility and protrusion into the channel reduce the channel thickness and elution is more rapid, although this is easily detected by measuring the channel void volume with an unretained probe. More subtle effects include surface roughness and membrane pore size, as demonstrated by **Figure 4**. Although the experimental arrangement, carrier sample chemistry and flow rates are the same for both experiments, the effect of the membrane is clear. The molecular mass of cytochrome c is only just greater than the membrane size cut-off (12 500 versus 10 000), and the delayed retention from the poor membrane may be attributed to a partial physical entrapment in the pores. For the poor membrane the pore size distribution may be particularly wide, leading to a greater proportion of the sample suffering excessive retention. The mean size and size distribution of the pores of the membrane are clearly an issue of importance. A simple solution is to choose a much finer membrane, for example a cut-off at 3000 or 5000 is appropriate for cytochrome c, but the pressure drop across the channel may be incompatible with high field flow rates needed for sufficient retention of small species. The

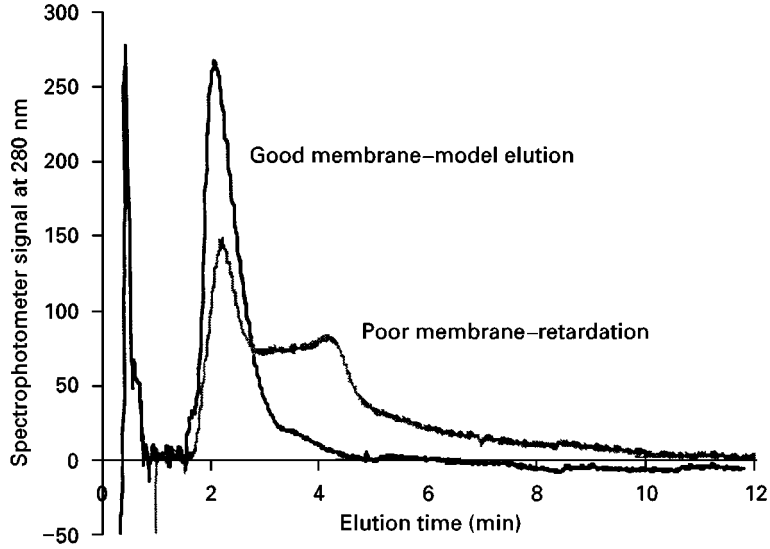

Figure 4 Superposition of two elution profiles for cytochrome *c* (0.82 mg mL^{-1}, 25 μL) in 0.05 mol L^{-1} 2-[*N*-morpholino]propanesulfonic acid ('mops') buffer at pH 6.2. The membranes are both regenerated cellulose, with cited 10 000 molecular weight nominal pore sizes from different suppliers. From Hecker, unpublished results.

presence of the membrane therefore determines the smallest-sized species capable of being retained in a Fl-FFF channel.

Such membrane effects have been used to advantage, however. Proteins have been characterized with a separation based on both standard FFF principles and enhanced retention for some species by sample–membrane interactions. This offers a remarkably wide scope for characterizing systems with subtle differences in physical sizes but dissimilar chemistries, but assigning peaks in the fractionation profile calls for a number of pure standards and calibration processes.

Of particular interest to protein science is the observation and quantification of protein–ligand or protein–protein interactions. Such an example is provided in **Figure 5** for the interaction between immunoglobulin IgC and an interacting ligand, polyglutamic acid, with the conjugate peak showing a small amount of free ligand. Quantifying such an interaction to measure the binding constant is a more difficult task. It is necessary to be able to produce fractionation profiles of the components as a function of concentration, implying that sample loss on to the membrane must be prevented. Furthermore, at least two from the protein, ligand or complex peaks must be well separated for quantification if the stochiometry is known prior to the experiment, otherwise all three must be resolved. This precludes

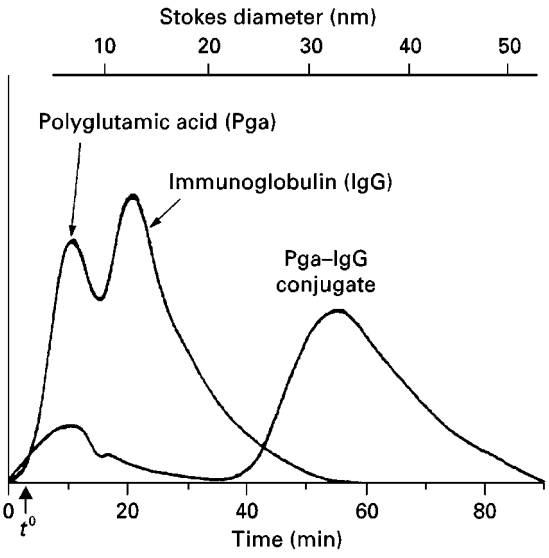

Figure 5 Elution profiles of the components of a protein–polymer ligand mixture, immunoglobulin IgG and polyglutamic acid, and their covalent conjugate. The fractionation of the conjugate suggests that a quantity of the polyglutamic acid remains unbound, and offers a method of determining the binding constants of such mixtures. (Reproduced with permission from Giddings JC *et al.* (1992) *Journal of Liquid Chromatography* 15: 1729 Copyright Marcel Dekker.)

many simple systems, for example bovine serum albumin (BSA)/anti-BSA, or ovalbumin/concavalin A, where the hydrodynamic sizes of these species are too similar for reliable quantification.

The application for protein interaction studies is limited to processes in which the interaction time is insignificant compared to the transport time, effectively making protein studies with a kinetic barrier to interaction difficult. Further, the use of FFF to investigate sample–sample interactions has been criticized, in that during transport dilution will occur so equilibrium in the FFF channel will be different to that of the mixing conditions. These limitations are clearly not relevant for rapid, near-irreversible interactions.

The opportunity for the study of protein shape by Fl-FFF is possible. Like other hydrodynamic methods, the information available from these methods renders them primarily as complementary methods to high resolution crystallography or magnetic resonance. Nevertheless, both theory and practice, discussed by Cölfen and Pauck, demonstrate that retention is a function of molecular shape (**Figure 6**), with the retention decreasing with the degree of asymmetry.

All these examples show that Fl-FFF is a powerful technique for protein characterization, as it is both very rapid and requires only microgram or smaller amounts of sample. Future potential can be seen in the quantification of interactions between proteins. However, potential factors affecting the results and possibly producing artefacts, such as membrane–sample interaction or sample shape, must be considered when interpreting the results.

Sedimentation FFF

The technique of sedimentation FFF balances the back-diffusion of the sample against sedimenting forces, a function of the sample's hydrodynamic diameter, density difference and the rotation rate applied. Sedimentation FFF offers significantly greater size-based sensitivity over Fl-FFF, with a corresponding greater resolution. The method is also free of the complications arising from the membrane required by Fl-FFF, although the possibility of electrostatic effects between the sample and cell cannot be ruled out. Unfortunately for protein applications, where the hydrodynamic diameters are of the order of a few nanometers and sample density is close to that of the buffering liquid, the rotation rate of the channel, and thereby the applied force field, must be high. None the less, successful application of the sedimentation FFF method to the characterization of biopolymers has been reported. The samples of interest tend to be among the larger biopolymers, and

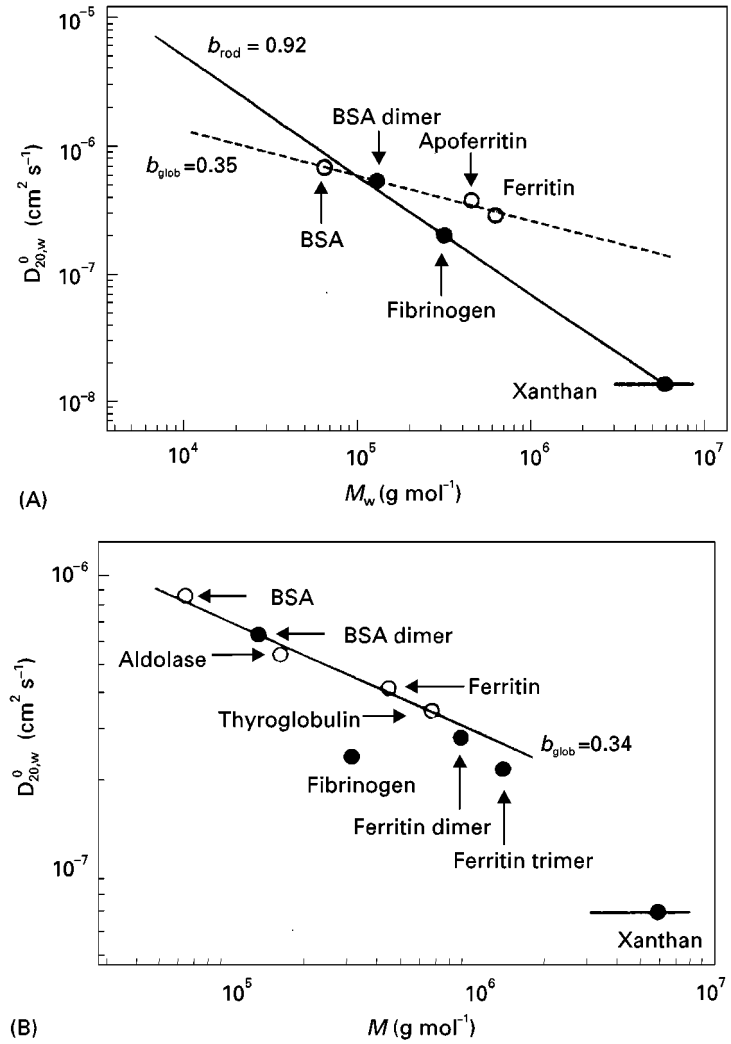

Figure 6 Temperature-corrected diffusion coefficients for a variety of proteins, using both analytical ultracentrifugation (A) and asymmetric FFF (B). The molecular weight–diffusion coefficient relationship is linear for the globular proteins, represented as open circles. Less spherical samples (filled circles) show a deviation from the linearity, with increasing deviation with eccentricity. (Reproduced with permission from Pauck T and Cölfen H (1998) *Analytical Chemistry* 70: 3886 Copyright the American Chemical Society.)

reported examples include DNA, proteoglycans, fibrinogen and myohemerythrin.

Thermal FFF

Thermal FFF, employing the Soret effect, is also suitable for the separation of biomolecules. Unfortunately, the thermodiffusion effect is extremely poor in water. The use of organic solvents restricts statements about the native state in aqueous-based buffer, and furthermore extensive conformational changes and even denaturation may occur which significantly restrict the range of applicable samples. Reported uses of thermal FFF for biological samples have been limited to the polysaccharides, dextrans, ficolls, pullulans and cellulose, and the starch polymers amylose and amylopectin, in dimethylsulfoxide as carrier liquid. Partially aqueous carriers have been investigated but it is the fraction in organic solvent that explicitly determines retention.

Electrical and Magnetic FFF

Electrical FFF is a subtechnique devoted to the fractionation of proteins, as reflected in the number of examples with protein applications. The narrow channel leads to high electrophoretic gradients across the cell, so samples with similar electrophoretic mobilities and differences in diffusion may be separated. As such, electrical FFF exists as a complement to electrophoresis. As early as 1972, a paper by Caldwell *et al.* first demonstrated the possibilities of

electrical FFF for the separation of albumin, lysozyme, haemoglobin and γ-globulin in buffer solutions at different pH.

Later, the performance of an electrical FFF channel with flexible membranes, a channel with rigid membranes and a circular channel for the separation of proteins was described. In these studies, human and bovine serum albumin, bovine γ-globulin, cytochrome *c*, egg white lysozyme and soluble ribonucleic acid (t-RNA) as well as denatured proteins were successfully separated. Unfortunately, the electrical field induces charge polarization of carrier liquid species, such that they migrated adjacent to the electrodes and then screen the electrical field. These early experimental configurations of electrical FFF utilized ion-permeable membranes separating the channel volume from the electrode compartments. These conditions led to difficulties in forming a homogeneous electric field, and from the late 1970s the technique entered a period of quiescence. Results published in the early to mid 1990s using conductive, rigid walls of either graphite or gold-plated glass, have allowed reproducible separations, while the addition of a redox couple in the carrier liquid, such as quinone-hydroquinone, reduced the polarization effects. Due to these delays in experimental development, electrical FFF is less mature than other FFF techniques.

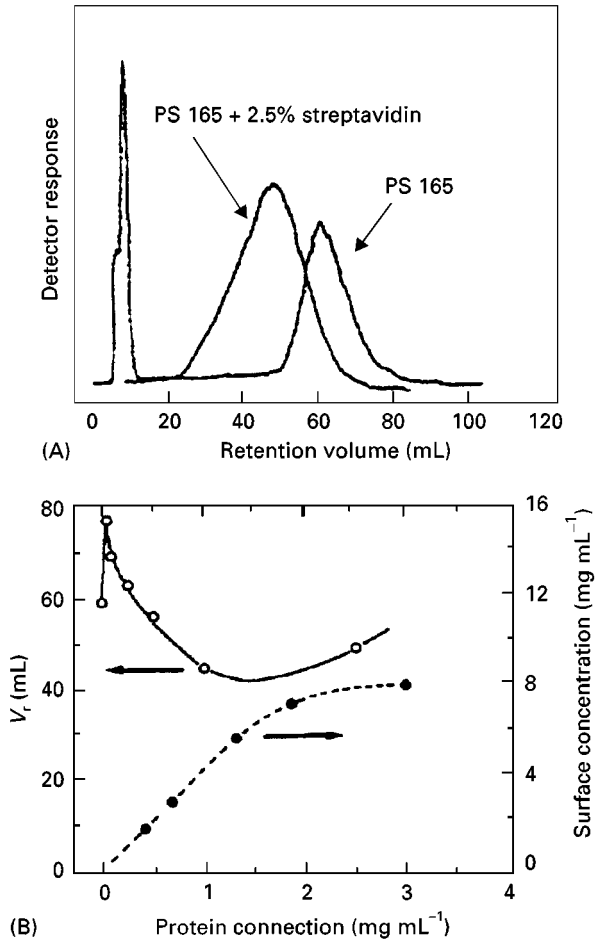

Figure 7 The coating of streptavidin on to a standard 165 nm diameter polystyrene (PS) latex bead affects the elution of the latex substrate by electrical FFF. Under pH 7.2 fractionation conditions the latex has a negative surface charge while the protein is isoelectric. The lower net surface potential is reflected in the poorer retention of the coated bead (A). The magnitude of this peak shift quantifies the degree of surface coating, as shown by the correlation in retention with the protein adsorption isotherm (B). (Reproduced with permission from Schimpf and Caldwell (1995) *American Laboratory* 27: 64–68.)

Electrical FFF is also well suited to measuring protein adsorption on to surfaces. The thin layer provides only subtle differences to the hydrodynamic size and net density, making flow or sedimentation FFF analysis difficult. However, the adsorption dramatically influences the surface charge and thereby influences both sample–field interaction and retention, as shown in **Figure 7**.

Although not formally FFF, dielectrophoresis in combination with fluid flow through an open chamber with interdigitated sinusoidally corrugated electrodes has been used for the separation of proteins and DNA.

A minor method, magnetic FFF, has been applied to study the retention behaviour of BSA in the presence and absence of nickel nitrate. In the presence of nickel ions, the retention time of the BSA sample was 6% higher with the magnetic field than it was without the field. Retention times reported for BSA samples both with and without a magnetic field did not differ in the absence of Ni (II). However, the application range of magnetic FFF for protein separations is very limited, and the method can only be applied in exceptional conditions.

Micropreparative FFF Applications

A variant of the FFF apparatus, the split-flow thin cell (SPLITT) permits continuous separation of milli- or even gram quantities of material. The apparatus is similar to a FFF cell equipped with both frit inlets and outlets. Initial configurations fed a mixture of large particles into the upper wall and carrier liquid into the base, while at the other end of the cell the liquid flowed out of two opposing exits. The ratio between the flows produces a hydrodynamic 'splitting plane' in the cell. During passage the larger particles could sediment sufficiently to exit at the other end of the SPLITT cell through the base, while smaller, less dense particles did not pass the splitting plane and eluted through the top. For protein applications, an electrical potential applied across such a cell in a range of buffers allows proteins with greater

electrophoretic mobility to pass the splitting plane. The separation of a mixture of model proteins by such a method has been reported. The relatively high throughput reported ($15\,mg\,h^{-1}$) makes this an interesting development for routine purification, but it requires a difference in protein *pI* of about two units as a necessary precondition for separation.

Miniaturization of FFF

There is a drive to produce the equivalent of hand-held devices for sample analysis based on the FFF principles, the chip laboratory. Advantages of such methods include the ability to analyse freshly sampled, or to undertake a number of simultaneous parallel analyses. For such miniaturized devices the injection volume is a significant proportion of the channel volume, with commensurate band-broadening problems, while theory predicts that some quantities, such as retention ratio and plate height, degrade with decreasing size. None the less, the reported developments for microfabricated electrical and dielectrophoretic FFF show healthy progression.

Concluding Remarks

The early development of FFF was hindered by the experimental complexity of the method and a focus on theory over practice. Over the last ten years, a number of simplifying experimental features such as the frit inlet–outlet system, and a fuller understanding of the theoretical background have led to a dramatic worldwide rise in the number of applications. It seems unlikely that more novel fields will be introduced into this family of techniques, but the subtlety of application is increasing. Methods and procedures are developing, from the analysis of simple proteins and mixtures, to protein aggregates, *proteins in complex matrices and increasingly fragile samples such as liposomes*, where the open channel has few, if any, real analytical competitors.

The other exciting branch of development is increased commercial application, where the FFF method becomes a 'black box' technique. Leading the way is the Fl-FFF method, but with the recent innovations in electrical FFF, the dominance of gel electrophoresis for protein analysis may be passing.

See also: **III/Proteins:** Centrifugation.

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Glycoproteins: Liquid Chromatography

See **III / GLYCOPROTEINS: LIQUID CHROMATOGRAPHY**