International Journal for Pharmaceutical Research Scholars (IJPRS)

V-3, I-4, 2014 ISSN No: 2277 - 7873

REVIEW ARTICLE

Field Flow Fractionation Technique- A Review

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Manuscript No: IJPRS/V3/I4/00423, Received On: 31/10/2014, Accepted On: 09/11/2014

ABSTRACT

Field Flow Fractionation (FFF) is a family of analytical technique developed specifically for separation and characterization of macromolecules, supramolecular assemblies and colloids. It combines the effect of laminar flow profile with physical field applied which is perpendicular to flow of carrier liquid. FFF is becoming popular as the researchers are learning its potential. The technique is also versatile and can be easily coupled with other analytical techniques like chromatography, spectrometry etc. Present review underlines the basic principle involved and theory of FFF along with its types like thermal FFF, flow FFF etc. Further the review elaborates its application to characterize natural, biological and synthetic polymers as well as proteins including analysis of lipid DNA complexes.

KEYWORDS

Field Flow Fractionation (FFF), macromolecules, Separation, Sedimentation FFF, Thermal FFF, Flow FFF, Electrical FFF

INTRODUCTION

In ancient times mankind most likely relied mainly on sedimentation to yield clear liquids. This means a long waiting period before a clear liquid could be decanted. Undoubtedly, filtration through some medium was fastest approach one could use to achieve the desired goal. Earlier, to achieve clear liquid focused is only on the separating visible particles but the fact is particles come in various sizes; some are so small that they are not visible to human eye without magnification. It is important to realize that there is no sharp discontinuity between very large molecules, colloid particles and macroscopic particles.[1] FFF was invented in 1966 by Prof. Calvin Giddings at University of Utah, Salt Lake City, USA. Commercial FFF systems have been available since the late 1980s.[3]

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FFF is family of analytical technique developed specifically for separation. The numerous variants in field type, instrument configuration, channel structure and operating mode make FFF uniquely adaptable to separate and characterize enormous of macromolecular and particulate materials. This versatility and adaptability is major strength of FFF making it potentially applicable within many scientific and technological disciplines that involve studies of polymers, bio particles and many additional building blocks of modern synthetic and natural materials.[18]

These separation techniques are combined with an appropriate detector(s) for the online or offline determination of various molecular characteristics. The most commonly utilized detectors with FFF are Multi-angle light scattering (MALS), differential Refractive Index (dRI), Ultraviolet and Visible (UV–vis), Nuclear Magnetic Resonance (NMR) and Fourier transform infrared (FTIR). MALS, dRI,

UV–vis, offer the advantage of online detection. Matrix assisted laser desorption time-of-flight mass spectrometry (MALD-TOF–MS) has also become a workhorse for macromolecular analyses.[4]

Principle and Theory

FFF is an elution based chromatography like method in which the separation is carried out in a single liquid phase. FFF is characterized by the use of an external field applied perpendicularly to the direction of sample flow through an empty, thin ribbon like channel (Fig. 1a). Due to the high aspect ratio of the FFF channel a laminar parabolic flow profile develop. Near the wall of channel flow velocity is zero than increasing to a maximum at the centre of the channel (Fig. 1b). The perpendicularly applied force drives the sample toward the accumulation wall. A counter acting diffusive force develops due to the concentration build up at the wall and drives the analyte back towards the centre of the channel. When the forces balance, steady state equilibrium is reached and an exponential analyte concentration profile is built up. Separation occurs because different analytes reside in different flow velocity zones.[4] The normal mode of separation, in which diffusion plays an important role in controlling component distribution across the channel is the most widely used mechanism.[5]

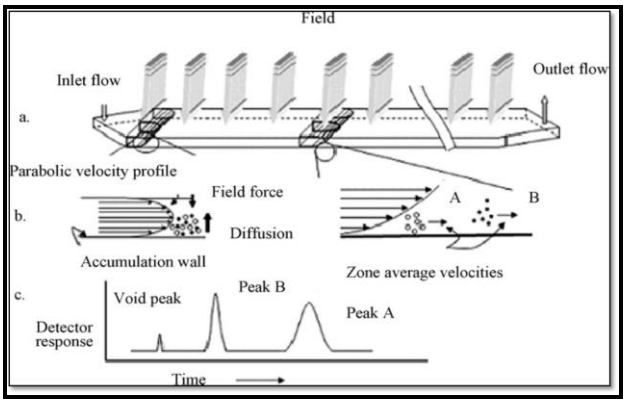

Figure 1: A Schematic Illustration Of The Basic Principle Of Normal Mode FFF Separation And A Typical Resulting Fractogram Are Shown In Fig. 1. The Fractogram Is A Detector Response versus Elution Time (Or Elution Volume) Curve. (A) Schematic Representation Of An FFF Channel Cut-Out, (B) Exploded Views Of The Normal Mode Separation Mechanism Of Two Components A And B (Faster Diffusing B Components Are Located At Higher Elevation In Faster Velocity Streamlines And Are Thus Eluted Earlier Than Slower Diffusing A Components) And (C) A Typical FFF Fractogram

Operation Mode

Three widely used modes that can be implemented in any FFF technique are as follows

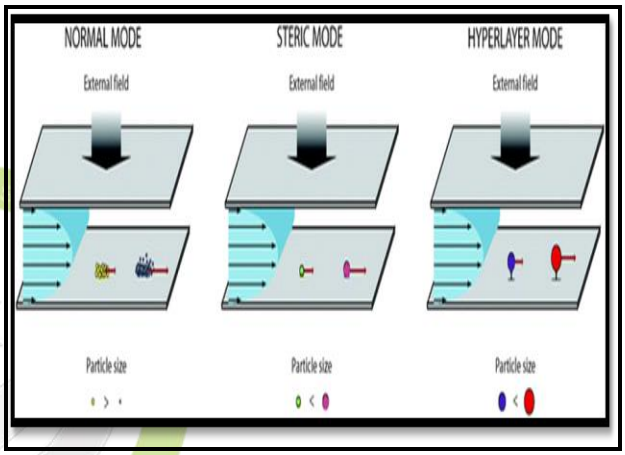

Figure 2: Different Mode of Field Flow Fractionation

Normal Mode

Normal mode also known as Brownian mode occurs when the analytes have a diameter smaller than 1 µm. In this mode, the analytes are pushed against the accumulation wall during the relaxation time. Then, as they are carried through the channel via the axial-flow, the analytes diffuse back towards the centre of the channel.[3] The analytes will equilibrate into a position based on their diffusion and the opposing cross-flow force. Smaller analytes have greater diffusion forces and will migrate further towards the channel's centre. Due to the laminar flow profile, this results in an elution order of small to large analytes.[6]

Reverse Mode (*Steric Mode/Hyperlayer Mode)*

The second separation mode is steric mode which occurs with analytes greater than 1 µm in

diameter. Once a molecule becomes larger, the diffusion forces are too small to play a role in the separation. In this case, an opposing force caused by the rebounding cross-flow from the accumulation wall "lifts" the particles away from the wall. The greater the diameter the more area the lift force has to push the particle back towards the centre of the channel. This result in an elution order of large to small particles.[19] An additional separation mode can occur in the FlFFF system called hyperlayer mode. This mode is conditional on the use of particularly high cross-flow rates with micron-sized particles and can be difficult to differentiate from steric mode.[5]

Most polymers are separated by the normal mode mechanism because their dimensions are less than 1µm.[6]

Instrumentation and Techniques

Fields in FFF

Various fields have been used in FFF depending on the nature of the material to be analyzed. The criteria for an effective field are sufficient strength and selectivity to achieve separation and ease of implementation. Each type of field interacts with a different physicochemical property of the analyte. Typical fields include a cross-flow stream, temperature gradient, electrical potential, centrifugal force, gravitational force, dielectrophoretic and magnetic fields. These give rise to several FFF techniques including flow (FlFFF), thermal (ThFFF), electrical (ElFFF), sedimentation (SdFFF), gravitational (GrFFF), dielectro-phoretic (DEP-FFF) and magnetic (MgFFF) field-flow fractionation. Analyte retention and separation in these different FFF techniques are achieved according to different analyte properties such as size, thermal diffusion and charge, density, mass and magnetic.[5] Field strength is the most important experimental condition in FFF because it has a strong effect on the resolution and separation time. The field strength is changed according to a decay function over the course of the analysis. Field programming is useful when studying broad distributions of molar masses or particles. It is used to optimize resolution and analysis time and enhance the detectability of fractionated analytes. Several types of field programming have been used including linear, parabolic, exponential and step-wise decay functions. Field programming in FlFFF and ThFFF is implemented by reducing the cross-flow rates and temperature gradients respectively. Generally, for lower mass and smaller particles higher field strength is required.[18]

Channel Geometries in FFF

The FFF channel is constructed by clamping a thin spacer (usually of Mylar or polyimide) with the desired geometric cut-out between two blocks with flat surfaces. The block material must be compatible with the carrier liquid and transmit the applied field. The ribbon-like channel in Fig.3 is the most commonly employed channel geometry. With this ribbon-like structure a perpendicular orientation of most fields can be achieved because diffusion is a slow process.

The channel thickness (w) must be small enough that the sample reaches equilibrium in a reasonable short time. The length of the channel (L) needs to be long enough to allow adequate retention time differences between the analytes. Typical channel dimensions are thicknesses of 50–500µm, breadth of 2 cm and tip-to tip lengths of 25–90 cm.

Triangular end pieces are used to allow smooth inlet and outlet flow. This type of rectangular channel can be considered as two parallel plates between which the parabolic laminar flow velocity can easily develop. Trapezoidal shaped channels (inlet breadth is greater than outlet breadth) as proposed by Litzen and Wahlund are used in AsFlFFF to allow more constant cross-flow along the length of the channel.[5]

In flow field fractionation below spacer ceramic frit or membrane is placed. A cross-flow is applied as the field perpendicular to the channel flow. Cross-flow enters the channel through the porous frit on the top wall and exits the channel through membrane as shown in the figure 3.

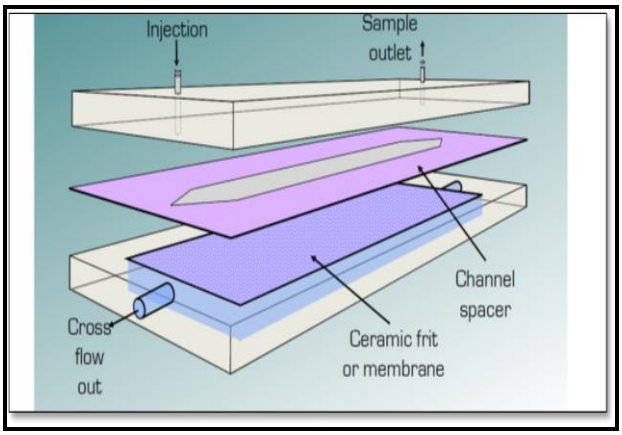

Figure 3: Schematic Representation of Channel of FFF

Working

Field flow fractionation has two separate inlets for the sample mixture and a carrier and two outlets for the bifurcated/separated samples. The carrier stream compresses the sample particles against one wall and the field perpendicular to the flow drives sample particles with some minimum interaction across the carrier stream interface where they elute from the opposite outlet. Particles that do not exhibit this minimum interaction with the field continue in the original sample stream and elute from the outlet on the same side as the sample inlet port then sample collected by fraction collector.[7] Sample and carrier fluid is drive by pump. Samples is injected using a loop injector normally Rheodyne Injector. Carrier liquids used in the separation may be distilled and deionised water with buffer.

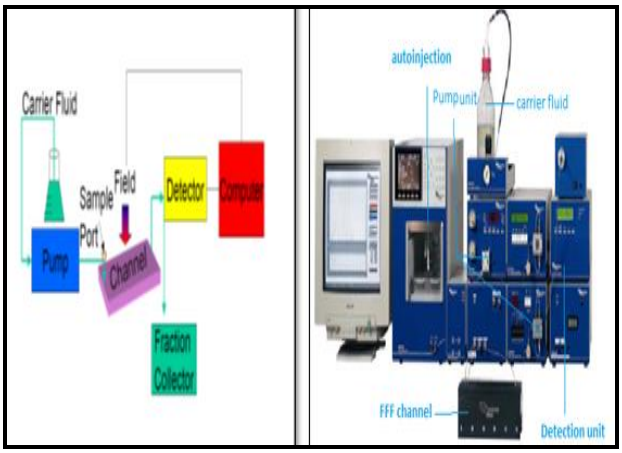

Figure 4: Field Flow Fractionation Schematic Representation and System

Eluted samples can be monitored by detector like a UV detector, MALSD, RI detector. Two PC-compatible computers are connected to the detectors for data acquisition and analysis. Flow rate is continuously measured with the aid of model balances, which is connected to computer via serial ports.[8] Fractogram is obtained by recorder which is represented by a plot of detector response versus time as shown in figure 1c.

Field Flow Fractionation Methods

Different FFF sub techniques result from the application of different types of field or gradients.

Sedimentation FFF

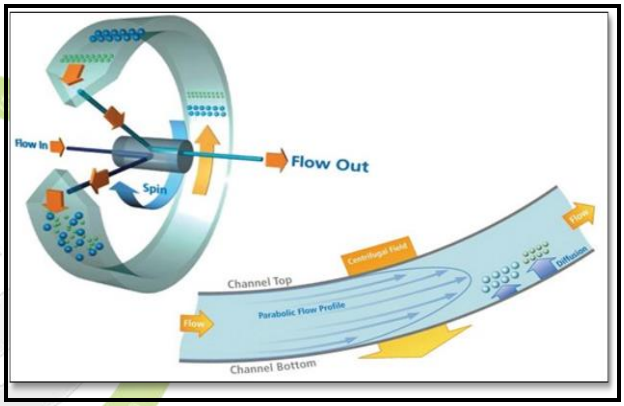

Figure 5: Schematic Representation of SdFFF Instrument

In SdFFF the separation force is established by a centrifugal field which is generated by spinning the complete circular SdFFF channel at a high rate. The bigger and denser particles are forced more in the direction of the bottom wall (outer wall) than the smaller particles.[2] Thus the bigger particles are eluted later from the SdFFF channel as they are located in slower streamlines in the channel than the smaller particles which are located in faster stream lines. Typical spinning rates which are used in SdFFF go up to 2500 rpm. To ensure a high resolution over a broad size range spinning gradients is used for the SdFFF separations.[8]

Thermal FFF

In ThFFF the separation force is established by applying a temperature gradient. The top wall of

a ThFFF channel is heated up and the bottom wall of a ThFFF channel is cooled. The typical temperature difference between the cold and the hot wall used in ThFFF is ranging between 20 and 100°K. This technique is ideally suited for the characterization of polymers, gels and nanoparticles.[10]

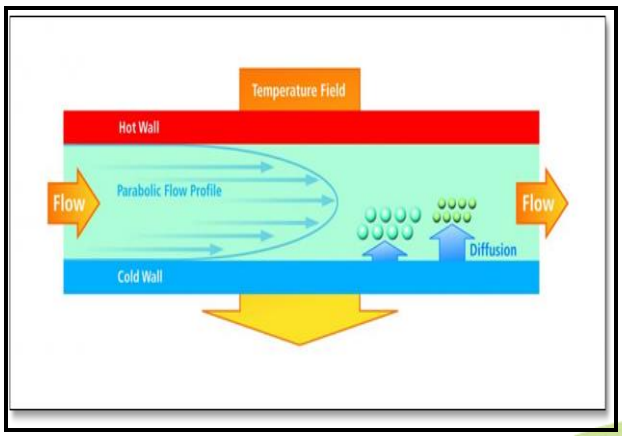

Figure 6: Schematic Representation of ThFFF Instrument

Flow FFF

Two types of FlFFF are considered in this symmetric flow field-flow fractionation (FlFFF) and asymmetrical field flow fractionation (AsFIFFF).

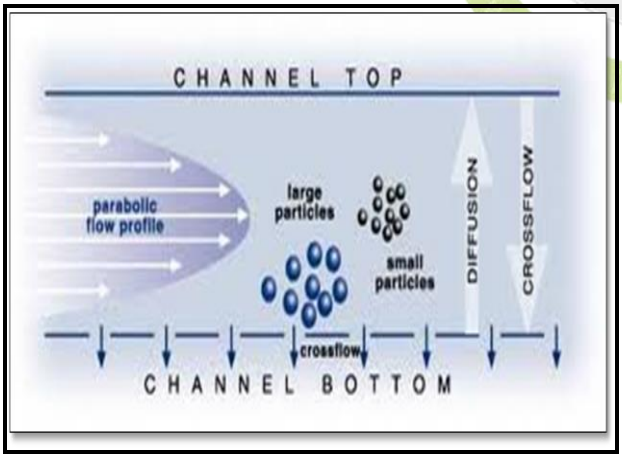

Figure 7: Schematic representation of FlFFF instrument

Symmetric FlFFF was introduced in 1976 by Giddings. In this first form of flow FFF the channel spacer is clamped between two parallel plastic blocks fitted with porous ceramic frits (2–5µm pores) in each wall.[5]A cross-flow is applied as the field perpendicular to the channel flow. Cross-flow enters the channel through the porous frit on the top wall and exits the channel through an ultrafiltration membrane, overlaying a second porous frit at the bottom wall (the accumulation wall). AsFlFFF differs from FlFFF in that the channel has only a single permeable wall (the accumulation wall). The upper porous wall is replaced by a solid wall that is impermeable to the carrier liquid. A single channel inlet flow is split into the channel flow and the cross-flow. AsFlFFF having the following advantages over FlFFF i.e. simpler construction and the ability to visualize the sample through a transparent upper wall.[13]A schematic representation comparing FlFFF and AsFlFFF is shown in the figure.8.

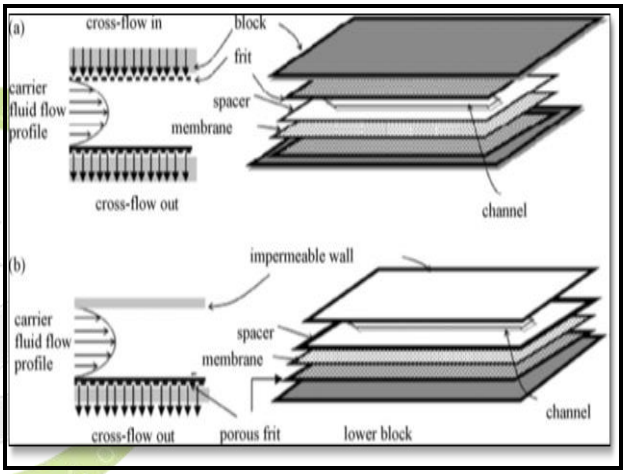

Figure 8: Schematic Representation of (A) Symmetric (FlFFF) and (B) Asymmetric (AsFlFFF) Channel Structures

Electrical FFF

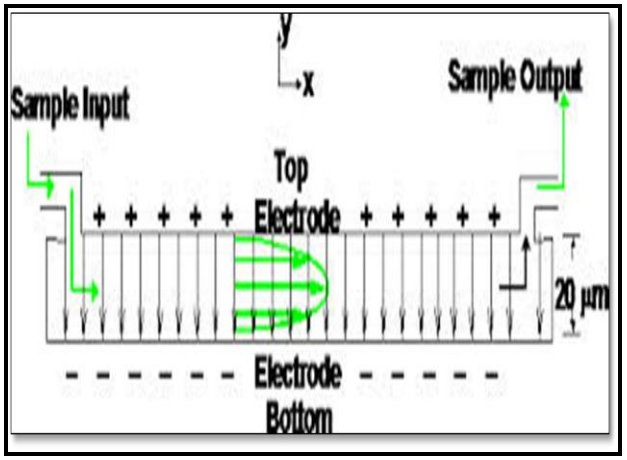

Figure 9: Schematic Representation of EFFF

EFFF is a molecular separation technique first described in 1972, when its feasibility demonstrated using various proteins. EFFF as mentioned previously is not a direct field separation technique but rather relies on an electric field perpendicular to the direction of separation (perpendicular to flow direction) to perform the separation function as shown in Figure.9.[5] The separations are performed in a low-viscosity liquid (typically an aqueous buffer solution) which is pumped through the separation channel. The EFFF process is based on controlling the relative velocity of injected particles by forcing them toward the wall of the channel. Particles with high electrophoretic mobility or zeta-potential will pack more closely to the wall, while particles of lower zeta-potential will diffuse more into the flow stream.[10]

Advantages

FFF can overcome some of the common limitations of traditional chromatographic techniques in several ways:

1. There is no stationary phase; therefore, there are no sample breakthrough effects or sample loss due to adsorption to the stationary phase.
2. The upper limit of FFF extends to the 10^9 Da molecular weight range and micron-size particles, thus providing effective separation of microgel components simultaneously with solubilized polymer.
3. Shear degradation is minimized.
4. Materials can be separated with high resolution over a wide size range from 1nm to 100 µm.
5. Different types of samples can be accommodated because of the physical simplicity and stability of FFF systems and the ease of adjusting experimental conditions.
6. The analysis of fragile analytes such as protein aggregates, supramolecular assemblies and whole cells is possible due to the mild operation conditions.[5]

Limitation

1. It is expensive due to several different instrumental systems are required to cover the range of potential applications of FFF.
2. Guidance and education is required to choose suitable instrumentation and

   condition to best solve specific problems.[5]

Advance in Field Flow Fractionation

Hollow-Fiber Flow-FFF (HF5) is almost as long as flat channel Flow-FFF. HF5 uses a completely different channel geometry based on a polymeric or ceramic hollow-fiber with porous walls as a cylindrical channel. The fibres are produced in large quantities for water purification. They have ideal properties for FFF as they allow a high flux, are mechanically stable, and have been shown not to interact with most types of samples, specifically proteins.[13]

High separation efficiency is main advantage of HF5.This benefit is based on specific properties of the HF5 separation mechanism. Plate numbers in Flow-FFF increase with the cross-flow velocity and retention time. Higher efficiency is achieved by using more cross-flow, which concentrates the sample closer to the membrane. This helps to prevent overloading and sample aggregation or absorption. Consumption of carrier solvent is lower for the same reason, reducing time and effort to prepare buffers plus reduce disposition of the waste. The HF5 channel cartridge is rugged and has a life time exceeding 100 injections. For most users this will allow to use the same cartridge for one or two weeks or even longer.[19]

Application

FFF can be used for separation and characterization of a broad range of analyte including protein, polysaccharide, macro-molecules, microgel, nanoparticle, environ-mental pollutant, polymer. Coupling of FFF with MALS substantially amplify the analytical information obtained from FFF. A list of pioneering applications of FFF in the analytical field is as follow.

Colloids Separation and Characterization

Colloids gained over the years a great interest in industrial and academic researches as they considered sources of environmental pollution. Colloids including mineral derived colloids, humic acids, paper mill effluents, soil colloids. These colloids are considered as the main sources of environmental pollution. Known by their intensive black colour, high molecular weight particles are of a great concern for environmental and health researches as they revealed their effect on human mortality in big cities. FFF is a high resolution technique for the separation of colloidal matter into well-defined particle fractions. Colloids are separated and characterized by thermal FFF and also by SdFFF coupled with OMT (optical multiwavelength techniques) to obtain element composition profiles for each size fraction i.e. elution technique providing a sequence of mass or size-differentiated fractions. The FFF process is usually initiated by injecting a small sample, usually suspended in a volume of only 5-50 µl, into a stream of carrier liquid whose composition is chosen to best stabilize and least disrupt the particles suspended in the sample volume. The sample is then swept by the carrier liquid into an FFF channel where the fractionation takes place and then into a detector (such as an HPLC UV detector) where the fractionated components are detected. The entire process typically requires 5-30 min.[17]

Monoclonal Antibody Fractionation

Proteins especially monoclonal antibodies (MABs) have become increasingly important in pharmaceutical work. However, there are some important differences between conventional, chemically-synthesized drugs and proteins. Because of the complex and weak structure of proteins even a slight change in conditions such as pH value, temperature or mechanical stress may lead to aggregation and a loss of activity and/or stability.[20] A separation of MABs before analysis is preferred because a detailed investigation of a fraction would be much easier and provide a better insight into the physical and chemical properties compared to an analysis in the presence of a coexisting protein. AsFFF is a well-established preparative tool for the separation of MABs. A major advantage of AsFFF is we can use the formulation buffer of the protein as the mobile phase because separation is independent of the ionic strength of the buffer. FFF process is initiated by injecting a small sample, which is suspended into a stream of carrier liquid then sample is separated in FFF channel by using perpendicularly applied field. Fraction is collected after elution by fraction collector and then detected by UV detector. AsFFF is a useful method for analytical and preparative separation of protein aggregates since the separation can be performed in buffer or even pure water, thus facilitating subsequent activities.[13]

Liposomes Characterisation

Liposomes and vesicles have elicited great interest in a number of applications ranging from targeted drug delivery systems to cosmetics. One of the most important parameters of a liposomal formulation is its size distribution, tends to be in the range of 80–200 nm.[21] The combination of a cross-flow FFF and MALS instrument is ideally suited to the absolute characterization of particle sizes within this range. Because of the absence of a stationary phase FFF is able to separate a sample for subsequent light scattering analysis without the associated with size exclusion columns. The MALS instrument provides actual size measurements directly from the angular variation of the light scattered by the particles. In this fashion the distribution of liposome sizes may be quickly and accurately determined.[13]

Characterization of Sodium Alginate Used in Food

There has been considerable interest in recent years in the development of suitable test methods to characterize foods that have been irradiated to prolong shelf life. This note describes work undertaken on a grade of sodium alginate used as a food thickening agent using the MALS detector in conjunction with FFF to determine the effect of gamma irradiation on the molecular weight distribution. Sodium alginates

are polysaccharides widely used as a food additive or as a sterile wound dressing. The molecular weight properties of the alginate contribute directly to the end-use performance of these products. When used as a thickening agent. MALS can be determined if any changes in the size and the mass in the fraction collected from FFF after separation.

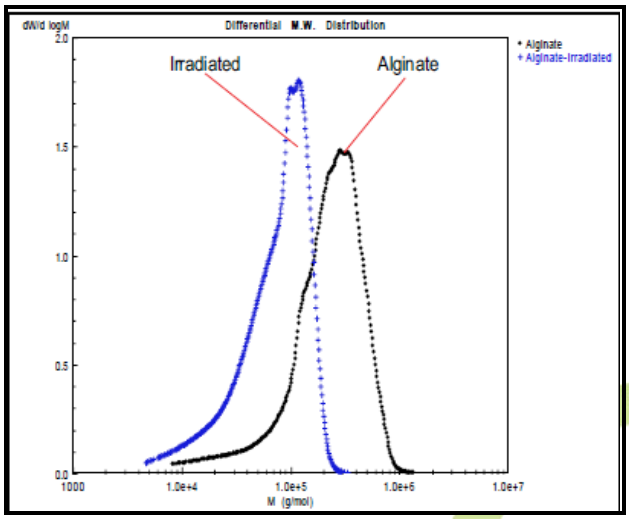

Figure 11: Differential Molecular Weight Plots for Sodium Alginate Before and After Irradiation

By plotting the log of the size (radius) as a function of the log of the mass (molecular weight), the resulting slopes will reveal whether the molecule is a sphere (slope of about 0.33), random coil (slope of 0.5-0.6) or a rod (slope of 1.0). From the plots shown in Figure we see that the natural, unradiated alginate has a compact, sphere-like structure. The irradiated sample shows that the molecules have opened to nearly rod-like structure with their slope of about 0.88.The radiation dose given to the sodium alginate caused the molecule to change from a fairly dense, sphere-like cross linked structure to a rod-like conformation. This suggests that the cross-linking bonds are damaged or destroyed by the irradiation, causing the molecule to extend and/or expand.[13]

Characterization of Cross Linked Polymers

Cross-linked polymer microparticles generally called microgels, have found extensive use in the coatings industry because of their utility in improving the rheological properties of paints as well as the performance of films.[5]Microgels are commonly prepared by emulsion polymerization in aqueous media. As is true of all polymers, the properties of microgels are tied closely with their molar mass and size. Transmission electron microscopy (TEM) along with field flow fractionation (FFF) is now routinely used to measure the size and molar mass of microgel dispersions. [15]

Characterisation of Synthetic Rubber: Polybutadiene

Synthetic elastomers have replaced natural rubber to an astonishing degree and account for more than 70% of the rubber used today. In the United States alone, 5 million tons of synthetic rubbers are produced annually. The principal synthetic rubber elastomer is a copolymer of butadiene and styrene. The latex form of rubber and synthetic elastomers has applications in carpet and gloves and coagulated latex is used for the production of tires and mechanical goods. It is of critical importance to know the absolute molar mass and its distribution, as well as to gain insight into the conformation of synthetic rubber which is indicative of the product's end-use performance. Typically, polystyrene standards are used to estimate the molar masses of these polymers in FFF but by using a MALS detector, standards and column calibration are no longer needed. Here, the absolute molar mass and polydispersity, as well as the rms radius of two synthetic rubber samples were measured directly using FFF combined with a MALS. Figure shows a logarithmic plot of the molar mass versus the radius of the two synthetic rubber samples. The slope of such a plot is indicative of the shape of a polymer.

A slope between 0.5 and 0.6 is usually found for linear polymers with a random coil confor-mation, while spheres have a slope of approximately 0.3. The values obtained for polybutadiene (0.25) and for the butadiene/ styrene copolymer (0.38) indicate that this polybutadiene is more branched than the styrene/butadiene copolymer.[12]

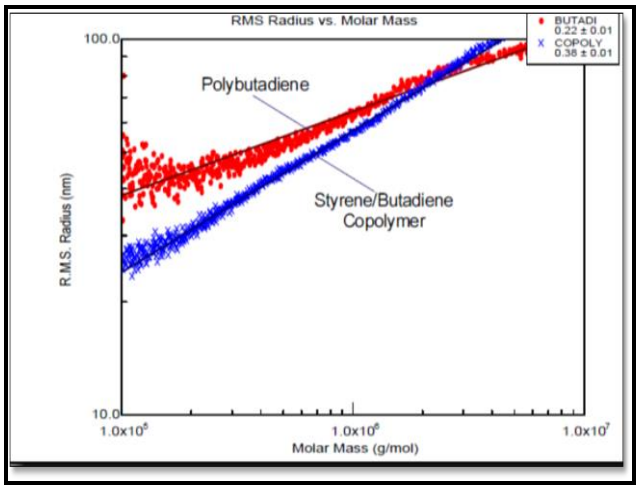

Figure 12: RMS Radius versus Molar Mass (Conformation Plot) for Two Samples. The Slope is Indicative of The Conformation (Rod, Coil or Sphere) of The Molecule

Characterization of Nanoparticles

AsFFFF in combination with MALS is one of the most promising methods for separation and quantification of nanoparticles (NPs) in complex matrices. For this study a MALS detector was coupled online to an AsFFFF system. In addition to the MALS detector different concentration detectors (RI-/UV-detector) and a fraction collector can be used. In a first step, it is possible to separate a sample into fractions of different sizes by using AsFFFF and then characterise them by their particle size distribution (MALS). After a successful separation, individual peaks of the fractogram can be collected and analysed by element specific techniques like inductively coupled plasma mass spectrometry (ICP-MS). Thus, a capable method is obtained to separate, characterise and identify a broad range of NPs.

Analysis of Cationic Lipid-DNA Gene Carrier Complexes

Cationic lipid-DNA complexes have shown an ability to facilitate the delivery of heterogonous DNA across outer cell membranes and nuclear membranes (transfection) for gene therapy applications. While the size of the complex and the surface charge (which is a function of the lipid-to-DNA mass ratio) are important factors that determine transfection efficiency, lipid-DNA complex preparations are heterogeneous with respect to particle size and net charge. This heterogeneity contributes to the low transfection efficiency and instability of cationic lipid- DNA vectors. Efforts to define structure-activity relations and stable vector populations have been hampered by the lack of analytical techniques that can separate this type of particle and analyze both the physical characteristics and biological activity of the resulting fractions. In this study, we investigated the feasibility of FlFFF to separate cationic lipid-DNA complexes prepared at various lipid-DNA ratios. The compatibility of the lipid-DNA particles with several combinations of FFF carrier liquids and channel membranes was assessed. In addition, changes in elution profiles (or size distributions) were monitored as a function of time using on-line UV-vis, MALS, and RI detectors. MALS detected the formation of particle aggregates during storage, which were not observed with the other detectors. In comparison to population-averaged technique such as photon correlation spectroscopy, FlFFF allows a detailed examination of suitable changes in the physical properties of nonviral vectors and provides a basis for the definition of structure-activity relations for this novel class of pharmaceutical agents.[14]

Other Applications[18]

1. Flow FFF (FlFFF)

A. Symmetrical F4:

i. Determination of diffusivity and separation of intact proteins.

ii. Molar mass determination of intact, ultra-large proteins and protein complexes in combination with light scattering.

iii. Separation of DNA at different conformation and measurement of diffusion coefficients.

iv. Separation of cationic lipid–DNA complexes using online UV, multi-angle light scattering and refractive index detectors.

v. Determination of virus diffusivity.

vi. Size-characterization of the tobacco mosaic virus with multi-angle laser scattering.

B. Asymmetrical F4:

i. Fractionation of protein mixtures.

ii. Study of protein interactions.

iii. Fractionation of plasmid fragments.

iv. Separation and quantification in one single analysis of tRNA in recombinant *E.coli*.

v. Convenient separation of recombinant granulocyte colony stimulating factor.

C. Hollow-fiber F4:

i. Fractionation of intact high-molar mass proteins.

2. Electrical FFF (ElFFF)

i. Separation of proteins.

ii. Micro-scale, sub- cellular particle separation for subcellular proteomics.

3. Sedimentation FFF (SdFFF)

i. Fractionation of DNA and smaller supercoiled plasmids in their native conformations.

ii. Separation and molar mass determination of the T2 phage.

iii. Fractionation of oligomeric aggregates of rod- shaped viral particles of nuclear Polyedrosis virus (NPV).

iv. Gentle fractionation of a wide variety of sub-cellular particles.

v. Purification and enrichment of neuron cell culture from a cortical cell suspension.

vi. Monitoring of inducted cell apoptosis in a human osteosarcoma cell line.

vii. High-resolution separation of bacterial cells from sediments, circulating blood on mouse ascitic fluid.

CONCLUSIONS

Field flow fractionation is powerful approach for separation and characterization of macromolecules. It also shows many evidences to retain enzyme activity and cell viability hence widely applicable in bioanalytical field. FFF in combination with other techniques such as MALS and MS can provide additional information.

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