A matter of size

Researchers are finding new ways to use field-flow fractionation for sizing everything from polymers to human cells.

BY RANDALL C. WILLIS

Field-flow fractionation (FFF), first described in 1966 by J. Calvin Giddings and his group at the University of Utah, represents a series of high-resolution chromatographic techniques used for the separation of high-molecular-weight particulates based predominantly on their size (~1 nm–100 µm). An alternative to techniques such as analytical ultracentrifugation and size exclusion chromatography, FFF has been used to separate and characterize particulate matter from solutions such as river water and adhesive production flow streams. But it is in the area of biomedical sample preparation where FFF is seeing rapid growth, with its use and development increasing in drug development and cell biology.

The technology

The basic FFF unit, available commercially from companies such as Postnova Analytics (*www.postnova.com*) and Tecan (*www.tecan.com*), comprises two plates separated by a spacer that forms a thin flow channel (Figure 1A). Because the channel is much wider than it is high, the buffer flow through the system is laminar, which is critical to its function. When sample is added to one end of the chamber, its particles become evenly distributed. A force such as gravity or electricity, which runs perpendicular to the channel flow direction, is then applied to the system. This initiates the separation of the particulates as the external force is counteracted by the natural diffusivity of the components in the sample. In most FFF systems, smaller particles respond to the perpendicular force less than larger particles and therefore sit further from the bottom of the channel.

When equilibrium is established between the perpendicular and diffusive forces, the external force is removed and the channel flow is initiated. The laminar buffer flow means that the linear fluid velocity at the chamber walls is zero and increases with distance from the walls. Thus, particles furthest from the channel wall meet the oncoming buffer stream first and evacuate the chamber earliest (Figure 1B). Heavier particulates are removed later.

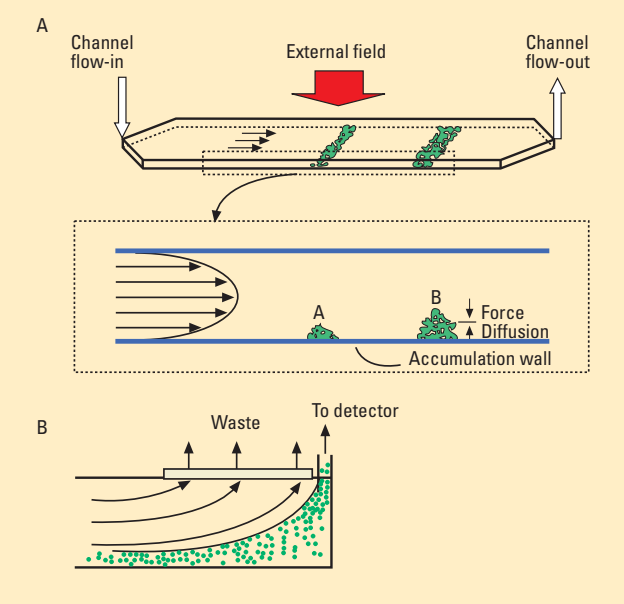

Figure 1. Separation without anxiety. (A) A sample and buffer are added through a frit at one end of the chamber, and the sample is forced toward the accumulation wall of the chamber by an external field. The distance of the sample particles from the wall is determined by the ratio of the external force and the diffusive force (magnification). (B) After removal of the external force, buffer flow resumes, and samples furthest from the chamber wall elute through a second frit. (Adapted with permission from Lee, H.; et al. *Anal. Chem.* **2001,** *73,* 837–843.)

FFF flavors

The precise nature of the particulates being studied dictates the nature of the perpendicular force, and several variations on the FFF theme have been developed to study different problems. These include sedimentation, electrical, thermal, and flow FFF.

In sedimentation FFF, the external force is applied through centrifugation, which directs the particles toward the bottom of the channel. Because of the nature of the sedimentation forces, this method is typically used to separate particles larger than 1 µm.

As might be expected, in thermal FFF, a temperature gradient is established by heating the plate on one side of the channel and cooling the other, and the particles migrate by thermal diffusion to the cooler plate. One of its key benefits is that it is one of the few methods that allow the researcher to determine the thermal diffusion coefficient of a polymer directly. Generally, thermal FFF is preferred for the analysis of synthetic polymers dissolved in organic solvents.

In flow FFF, one of the most common forms of the technique, the perpendicular force is supplied by a cross-flow of buffer that enters through a port on the top of the chamber and exits through a membrane on the bottom. The field strength therefore depends on the cross-flow rate. This method is typically used to separate particles in the range of <1 nm to 10 µm, making it ideal for studying colloidal suspensions in natural water samples.

Combining the benefits of electrophoresis with those of FFF, electrical FFF uses an

KEY TERMS: cell biology, drug delivery, genomics, process, proteomics, screening, technique

electric field generated between two graphite plates as the perpendicular force acting upon the particulates, such that they are separated not only on the basis of size but also by their electrostatic nature (zeta potential). These factors make the technique ideal for colloidal stability studies. Although the technology has been around for decades, the challenges of finding the right field strength without electrolyzing the water in the buffer have hampered its widespread use.

Cell separation

Relying on advances in establishing dielectrophoretic fields using interdigitated electrodes on a single chamber surface, Jun Yang and colleagues at the University of Texas M. D. Anderson Cancer Center (Houston) used dielectrophoresis FFF to separate living human cells (*1*). This separation is based on the principle that cell polarizability depends largely on cell composition, morphology, and type. Thus, under carefully chosen conditions, certain cell types will levitate within an electrical field, whereas other cell types will move to the bottom surface of a chamber. By examining the elution behavior of cells at various frequencies, Yang's group was able to separate a mixture of four leukocyte subgroups (T cells, B cells, monocytes, and granulocytes) according to their size, density, and membrane properties while maintaining cell viability.

In a similar study (*2*), Yang's group separated breast cancer cells from a population of normal T cells and stem cells. Such separations are critical in the early detection of cancer cells by methods such as flow cytometry or PCR. Furthermore, this technique can be useful in clearing stem cell populations of their cancerous contaminants when the disease is being treated by transplanting clean stem cells back into a patient after chemo- or radiation therapy.

More recently, Pierluigi Reschiglian at the University of Bologna and colleagues at several institutions performed their own version of cell separations. Rather than using dielectrophoresis FFF, they used sedimentation and flow FFF. The researchers were looking for ways to separate live and deactivated cells in a population of bacteria. Their goal was to isolate a pure population of deactivated bacteria for immunoprophylaxis—whole-bacteria vaccines—against diseases that are caused by or related to bacterial infection.

In one study (*3*), Reschiglian's group applied hollow-fiber flow FFF to a culture of deactivated *Vibrio cholerae*, the active agent that causes cholera. In part, the hollow fiber approach was selected because the channels are inexpensive enough to be disposable, an important criterion when trying to avoid cross-contamination of samples. Using the FFF apparatus, the researchers isolated the deactivated cells quickly and reproducibly, and by adjusting the buffer conditions with various surfactants, they were able to distinguish in some cases between two closely related strains of *V. cholerae*, which differ only by their lipopolysaccharide coats.

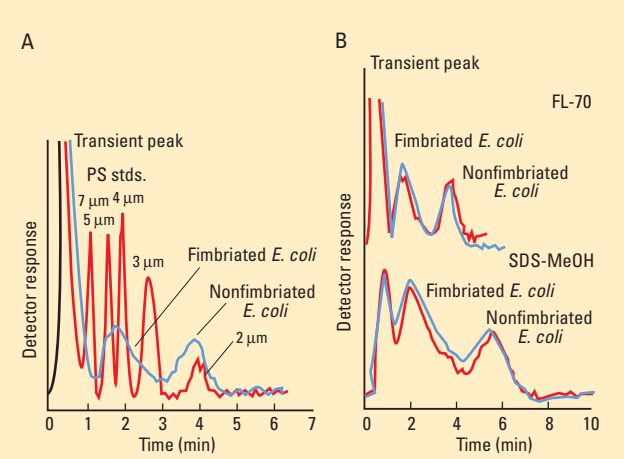

Figure 2. Bacterial separation. (A) Using asymmetric flow FFF, fimbriated and nonfimbriated *E. coli* are separated, and their sizes are compared with internal (PS) standards. (B) The retention profiles of the bacteria change when two different carrier solutions are used: FL-70 and SDS-methanol (MeOH). (Adapted with permission from Reschiglian, P.; et al. *Anal. Chem.* **2002,** *74,* 4895–4904.)

In a second, related study (*4*), the same researchers used gravitational and flow FFF methods to separate and identify different strains of deactivated *E. coli*. Unlike the *V. cholerae*, the *E. coli* strains differed by the presence of fimbriae, rod-shaped proteinaceous protrusions on the surface that affect how and when the bacteria will bind the epithelial cells of the human gut. Both FFF methods successfully separated the various strains and samples (Figure 2). Because gravitational FFF relies solely on the precipitation of the cells due to gravity (a low-tech version of sedimentation FFF), it takes appreciably longer (15–30 min) than flow FFF (<1 min), but it offers the benefit that it can be performed with any standard HPLC setup and does not require specialized equipment.

Macromolecule separation

Usually, however, researchers are not looking to use FFF to isolate large cells from a buffer solution. Rather, they are typically trying to separate macromolecular complexes from smaller proteins or salts. Such was the case in 1996, when Giddings and Ping Li used a membrane-modified flow FFF technique to isolate lipoprotein complexes and pharmaceutical colloids from blood plasma (*5*). The naturally high abundance of proteins in blood plasma often complicates measurement of colloidal materials that might be important indicators of health. Thus, the researchers placed a low-molecular-weight-cutoff membrane across the floor of the flow chamber such that proteins <7 nm would flow through the membrane during the perpendicular flow phase of the separation, leaving behind the macromolecular complexes. Using this system, the researchers were able to isolate HDL, LDL, and VLDL from plasma, as well as intravenous emulsions of liposyn II, a fat, and amphotericin B colloidal dispersion, which is used as a pharmaceutical carrier.

More recently, researchers at the Colorado School of Mines and the University of Colorado Health Sciences Center applied flow FFF to the preparation of substrates for gene therapy (*6*). Because the use of viral vectors for gene transfer has been plagued with concerns over patient safety (see "Gene Therapy: Hope in a cautionary tale", *Modern*

Drug Discovery, January 2003, pp 23–27), researchers have tried to use cationic lipid particles to deliver DNA plasmids to target cells. Unfortunately, the heterogeneity of these lipid–DNA complexes appears to interfere with their transfection efficiency. Thus, the Colorado researchers used flow FFF to isolate and subsequently characterize the complexes.

The researchers found that the FFF migration of the plasmid DNA depended on the nature of the molecules with which it interacted. DNA complexed with cationic cobalt was 22% smaller (migrated farther) than free DNA (Figure 3). This is due to the compaction of the DNA molecules as the negative charges of the phosphodiester backbones are neutralized by the cation. DNA mixed with the cationic lipid lipofectamine, by contrast, was 21% larger than naked DNA, indicating the formation of lipid–DNA complexes. The researchers also noted that complexes incubated for long periods appear to aggregate with time, which might help to explain the concomitant decrease in transfection efficiency.

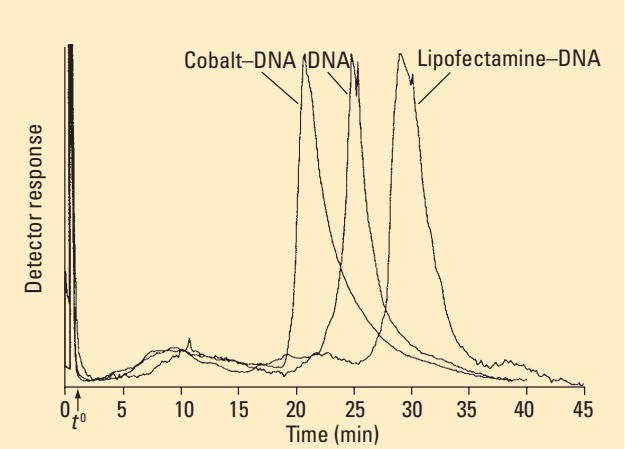

Figure 3. Macromolecule fractionation. Researchers used flow FFF to characterize plasmid DNA and DNA complexes. (Adapted with permission from Lee, H.; et al. *Anal. Chem.* **2001,** *73,* 837–843.)

FFF forecast

Although much work must still be done to optimize the technology for specific applications, the different FFF methods are making inroads into various chemical and biomedical analytical streams. With further development on the academic and industrial sides, the use of FFF is sure to expand in the future.

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