

# Particle Size Analysis of Dilute Environmental Colloids by Flow Field-Flow Fractionation Using an Opposed Flow Sample Concentration Technique

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**An on-line concentration method has been developed for the analysis of dilute suspensions of macromolecules and particles by flow field-flow fractionation (FFF). This method, termed opposed flow sample concentration (OFSC), uses two opposing flowstreams to focus sample into a narrow band near the inlet of the flow FFF channel. Large sample volumes (up to 1 L) have been successfully loaded, concentrated, and separated in a 1 mL volume flow FFF channel. Concentration factors as high as  $10^5$  have been achieved. The effectiveness of OFSC flow FFF was demonstrated using mixtures of monodispersed standards such as proteins and polystyrene latex beads. Retention times, peak areas, and resolution are comparable for a 10 mL and a typical  $10\ \mu\text{L}$  injection volume. The applicability of the method to environmental studies was demonstrated by a separation of river water colloids.**

Field-flow fractionation (FFF), which is a versatile high-resolution technique for the separation of colloidal matter into well-defined particle fractions,<sup>1,2</sup> is emerging as a preferred technique for the separation and characterization of environmental colloids.<sup>3–13</sup> Environmental applications of FFF have been spearheaded by Beckett and co-workers.<sup>7–16</sup> These investigators have examined a variety of water-borne colloids including mineral-

derived colloids, humic acids, paper mill effluents, soil colloids, and bacteria. They have had particular success in coupling FFF to other instruments (such as an inductively coupled plasma-mass spectrometer or ICPMS) to obtain element composition profiles for each size fraction.<sup>14–16</sup> Ancillary techniques such as ICPMS or electron microscopy are found to couple readily to FFF because the latter is an elution technique providing a sequence of mass- or size-differentiated fractions from a continuous outlet stream.

The FFF process is usually initiated by injecting a small sample, usually suspended in a volume of only  $5\text{--}50\ \mu\text{L}$ , 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  $5\text{--}30\ \text{min}$ .

The above simple procedure often becomes complicated for environmental samples because such samples tend to be dilute, so much so that common detectors are unable to sense the emerging components. In this case, the sample components must be concentrated before FFF separation begins. The concentration process usually requires extra steps in the experimental procedure. It may also lead to sample loss or coagulation, and it generally consumes more time than the FFF run itself. For these reasons, the concentration procedure may be a crucial step in limiting the speed and efficacy of the FFF analysis of environmental colloids.

Several procedures such as coagulation, centrifugation, and filtration have been used to concentrate the sample prior to separation by FFF.<sup>7,8,16,17</sup> An on-line procedure has been developed for sedimentation FFF (and could in principle be transferred to flow FFF) in which a stream of dilute sample is introduced at a sufficiently high field strength that the sample components collect as a thin almost stationary band at the head of the FFF channel. Once sufficient sample material has entered the channel, the field strength is reduced to a value suitable for FFF operation and the run is carried out.<sup>18</sup> An analogous concentration procedure has

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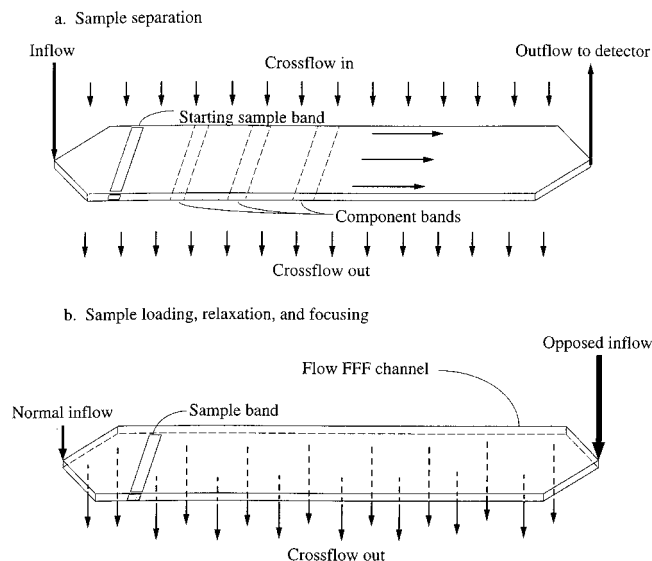


Figure 1. On-line concentration and separation in a flow FFF channel. (a) Flow paths during separation. (b) Flow paths during sample concentration (loading and focusing).

been reported using ionic strength rather than field strength.<sup>19</sup> As with most other procedures, these concentration processes may lead to sample loss and other changes, in part due to the high field or ionic strength used during injection.

We have designed an alternate method for on-line sample concentration in flow FFF<sup>20</sup> which has subsequently been used in the analysis of low molecular weight colloidal material in natural waters.<sup>5</sup> In this procedure, the sample can be loaded into the flow FFF channel at any desired field strength (cross flow rate), although there is a tradeoff between field strength and loading speed. The on-line nature of the sample injection and concentration means that no sample transfer steps are needed, thus reducing the risk of sample loss and at the same time simplifying the injection–concentration–separation procedure. In fact, the entire process from sample loading to FFF separation involves only flow switching, and is thus subject to automation.

The opposed flow sample concentration (OFSC) technique is based on the same principles of hydrodynamics that are used to focus and relax small sample injections in asymmetrical flow FFF.<sup>21,22</sup> (Asymmetrical flow FFF utilizes a nonpermeable rather than permeable upper channel wall, which makes sample relaxation more difficult. This problem is solved and at the same time the injected sample is narrowly focused by using opposing flows.) The opposed flow technique can be used in both symmetrical (standard) or asymmetrical flow FFF channels.

In the typical symmetrical flow FFF separation process, a flowstream enters on the left and exits on the right side of the channel as shown in Figure 1a. A superimposed cross flowstream (serving as a driving force) enters the channel through the slab of frit serving as the upper channel wall and exits through the combined membrane-frit layer forming the bottom channel wall.

(This cross flowstream, as it flows across the channel thickness, is responsible for the separation effect observed for flow FFF.<sup>1,2,23</sup>)

Using the OFSC technique, a sample can be loaded into an FFF channel and focused into a narrow band by using two opposing inflows at opposite ends of the channel as shown in Figure 1b. During this step, no flow is permitted to enter or exit through the upper frit wall. Therefore, the entering flow must exit the lower membrane-frit layer. Because flow generates a modest pressure drop across this layer and because there is virtually no pressure drop along the axis of the open channel, permeation through the membrane-frit layer is evenly distributed over the membrane area. The source of this outflow is two flowstreams. These streams enter at the ends of the channel and flow along the channel axis (one forward and one reverse in direction), all the time being depleted by the outgoing cross flow. The larger incoming stream, generally the opposed (reverse) inflow, will penetrate the greatest length into the channel because of the evenly distributed outflow. The two incoming flows, weakening as they penetrate into the channel, meet at the point where they both become fully exhausted. This is where the sample band accumulates.

Any particle introduced into either inflow stream will gradually migrate to the point where the streams meet, which thus becomes a focusing point (or line) for the accumulation of all sample particles. Particles migrate to this point because there is always a component of the flow vector, anywhere in the channel, pointed toward this focusing point. (The other major component of the flow vector is directed outward through the membrane; the particles cannot follow this path because the membrane is chosen to be nonpermeable to the particles.)

OFSC encompasses three processes: sample loading, focusing and relaxation, and flow stabilization. There is an overlap in the time frame over which these processes take place since the front edge of the sample is undergoing focusing and relaxation at the same time that more sample is being loaded into the channel. Hence, the sample loading time is defined as the time needed for the entire sample volume to be introduced into the channel; the focusing time is the time required for the sample at the tail end of the sample segment to be transported to the focusing point; and the sample concentration time is the summation of the loading and focusing times. Since relaxation of the sample to the accumulation wall takes place at the same time that the sample is being concentrated and the relaxation time is usually much shorter than the concentration time, an additional relaxation step is usually not needed.

For the introduction of small sample volumes, the sample enters the normal inflow inlet. After the sample enters, the two flows must continue for a sufficient time to bring about focusing and relaxation.

For large dilute samples, the loading step may require a significant amount of time. In most cases, time may be saved by loading sample through the opposed (reverse) inflow or both inflow streams, in which case sample can be loaded at ~5–10 mL/min.<sup>20</sup> The reduced loading time is offset by the increase in focusing time required for sample entering through the opposed flowstream (as a result of the greater distance between the focusing point and the opposed inflow inlet).

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Once the focusing point is chosen, it is readily realized by adjusting the ratio of the forward:reverse inflows. (For example, a 1:1 flow rate ratio would establish a focusing point at  $1/(1+1)$  or 0.5 of the length of the channel.) However, the absolute value of the inflows is subject to another tradeoff. High inflow rates will lead to faster sample loading, but the increased flux of liquid through the membrane may lead to sample deposition and loss at the membrane surface.

## THEORY

In the case of sample loaded through the normal inlet, the required concentration time,  $t_c$ , is given by<sup>20</sup>

$$t_c = \frac{V_S}{\dot{V}_F} + \frac{w^2}{6D} \ln \left[ \frac{L \dot{V}_F}{\Delta z (\dot{V}_F + \dot{V}_O)} \right] \quad (1)$$

where  $V_S$  is the sample volume,  $\dot{V}_F$  and  $\dot{V}_O$  are the forward and the opposed flow rates in the focusing process,  $w$  is the channel thickness,  $L$  is the channel length,  $D$  is the diffusion coefficient of the particle, and  $\Delta z$  is the concentrated zone width. The first term on the right-hand side represents the sample loading time, and the second term represents the focusing time. The sample loading time refers to the time taken for the entire sample volume to be pumped into the channel. The focusing time refers to the time needed to transport all sample components to the focusing point and can be derived by applying the retention theory of FFF.<sup>20,21</sup> It is evident from eq 1 that the shortest concentration time depends on the interplay between sample loading and focusing times. This will be discussed in more detail in a later section.

## EXPERIMENTAL SECTION

**Apparatus.** A schematic diagram of the flow FFF system is shown in Figure 2. The liquid flow paths during concentration and separation are denoted by the dashed and solid lines, respectively. An HPLC model 420 pump (ESA, Inc., Bedford, MA), pump 1, is used to drive the channel flow in the separation stage and the normal inflow in the concentration stage. An Eldex Metering CC-100-S-PK pump (Eldex Laboratories, Inc., Napa, CA), pump 2, is used to drive the cross flow during separation and the opposed flow during concentration. A variable pressure restrictor (Optimization Technologies, Bend, OR) can be connected to the channel flow or cross flow outlet to equalize back pressures and thus ensure that the flow rate at each outlet equals that at the corresponding inlet. (In this work, the pressure restrictor was connected to the channel flow outlet only.) Flow rates are measured with a buret and stopwatch. A more efficient, but also more costly approach, would be to use flowmeters or a balance to measure the change in mass with respect to time.<sup>24</sup> Two injection valves with 20  $\mu$ L and 10 mL sample loops (Rheodyne 7010, Cotati, CA) are incorporated into the system to allow fast and convenient switching between small and large volume injections. The 20  $\mu$ L loop was usually partially filled to 10  $\mu$ L. Valves  $V_1$ ,  $V_2$ , and  $V_3$  are three- and four-way valves from Hamilton (Reno, NV). Detection is achieved using a model 785A UV absorbance detector (Applied Biosystems, Ramsey, NJ) set at 254 nm. A PC-compatible computer is used for data acquisition and

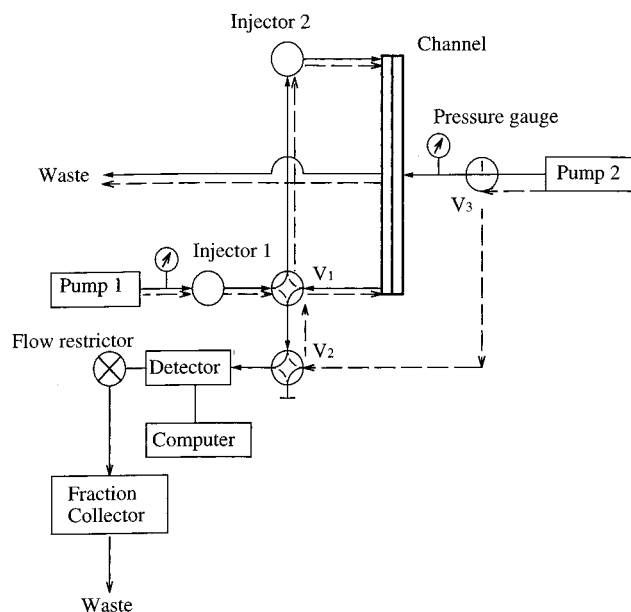


Figure 2. Schematic representation of the flow FFF apparatus. Solid lines indicate the flow paths during the separation step. Dashed lines represent the flow paths during the concentration step. Injector 1 has a 10 mL loop, and injector 2 has a 20  $\mu$ L loop which is partially filled to 10  $\mu$ L.  $V_1$ ,  $V_2$ , and  $V_3$  are three- and four-way valves.

analysis. Sonication was done utilizing a model W-225R (microtip) Sonicator Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY). A Hitachi S450 scanning electron microscope (Tokyo, Japan) was used to obtain micrographs of particulate samples at 20000  $\times$  magnification.

The flow FFF channel system consisted of a semipermeable membrane that served as the accumulation wall, a thin spacer with a defined channel form, and two Plexiglas blocks with inset ceramic frit panels. The ceramic frits had 5  $\mu$ m pores. The channel length,  $L$ , and breadth,  $b$ , were 26.5 and 2.0 cm, respectively. The thickness of the Mylar spacer was 254  $\mu$ m.

**Carrier Liquid and Membrane.** Preliminary investigations were carried out to determine the carrier liquid and membrane combination that would yield the best recovery of river sediment and kaolin standards. Carrier liquids used included 0.1% sodium dodecyl sulfate (SDS, Sigma, St. Louis, MO) and 0.02% sodium azide ( $\text{NaN}_3$ , Sigma, St. Louis, MO); 0.1% FL-70 (Fisher Scientific, Fair Lawn, NJ) + 0.02%  $\text{NaN}_3$ ; 0.05% dodecylamine hydrogen chloride ( $\text{C}_{12}\text{H}_{25}\text{NH}_2\text{HCl}$ , Lancaster Synthesis Inc., Windham, NH); and 0.05% sodium pyrophosphate ( $(\text{Na}_4\text{P}_2\text{O}_7) \cdot 10\text{H}_2\text{O}$ , Mallinckrodt, Paris, KY) + 0.05% sodium hexametaphosphate ( $(\text{NaPO}_3)_6$ , Johnson Matthey, Ward Hill, MA).

Various ultrafiltration and microfiltration membranes were used as the accumulation wall of the flow FFF channel. The former included regenerated cellulose (YM-30) and polysulfone (PM-30) from Amicon Inc. (now part of Millipore Corp., South San Francisco, CA). The latter included polypropylene (Celgard 2400) with an effective pore diameter of 0.02  $\mu$ m and aluminum oxide-coated polypropylene from Hoechst Celanese (Charlotte, NC) and 0.03  $\mu$ m polycarbonate with a hydrophilic poly(vinylpyrrolidone) (PVP) coating (Poretics Corp., Osmonics, Livermore, CA). The PVP used to condition the membrane in reproducibility studies was a gift from Poretics Corp.

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**Standards and Samples.** Polystyrene latex beads (0.091, 0.173, 0.304  $\mu\text{m}$ ) and kaolin were obtained from Duke Scientific (Palo Alto, CA). For stop-flow runs, the original sample suspension (10% solids) was diluted 100 times and 10  $\mu\text{L}$  of the resulting suspension was injected into the FFF channel. For concentration runs, 20  $\mu\text{L}$  of this 0.1% solids suspension was further diluted with 10 mL of carrier liquid. Protein samples (bovine serum albumin (BSA),  $\gamma$ -globulin, and thyroglobulin) were purchased from Sigma (St. Louis, MO). The river sediment standard (SRM 1645) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD). The concentrations of the river sediment and kaolin standards used in the stop-flow experiments were 1.7 and 1.9 mg/mL, respectively.

Water samples were collected from the Jordan River (Jordan Park, Salt Lake City, UT). Procedures for sample preparation are similar to that described by Beckett et al.<sup>8</sup> One gram of SDS and 0.2 g of  $\text{NaN}_3$  were added per liter of river water to prevent particle aggregation and bacterial growth. The river water was then stored in a refrigerator at 7 °C for a minimum of 24 h. A 100 mL aliquot of the river water was transferred to a beaker and stirred for a minimum of 1 h at room temperature. It was then allowed to sit for 20 min to settle out large particles. Twenty milliliters of supernatant was transferred to a small plastic tube and sonicated for 5 min after which 10 mL was introduced into the flow FFF channel.

**Procedures.** In a typical FFF experiment, the 5–50  $\mu\text{L}$  of injected sample is swept onto the head of the channel. The channel flow is then momentarily stopped by switching valve  $V_1$  shown in Figure 2. The cross flow is left on and drives the different sample components to their equilibrium positions near the membrane-accumulation wall. The stop-flow time is equal to the time needed for the cross flow to sweep out one channel volume. (The stop-flow time used in practice is  $\sim 30\%$  higher than calculated.) The channel flow is resumed at the end of the stop-flow period by returning  $V_1$  to its original position. The separated components are then eluted off the channel.

In a concentration run, valves  $V_2$  and  $V_3$  are switched so that pump 2 provides the opposing inflow. The sample can be loaded onto the channel through the normal inflow, the opposed inflow, or both inflows depending on the sample volume. (For the work reported here, a 10 mL sample was introduced through the normal inflow inlet). The sample migrates to the focusing point and is concentrated as a result of the transport (carrier) fluid selectively penetrating through the membrane and frit and exiting the system. In Figure 3a, the lines drawn in the channel depict the two opposing flows and are not intended to represent flow or particle trajectories. The concentration stage, which is comprised of sample loading and focusing, is followed by a flow stabilization step (Figure 3b) where  $V_1$ ,  $V_2$ , and  $V_3$  are switched, and the flow rates are adjusted to those used during separation. This step prevents undesirable pressure fluctuations which cause detector baseline disturbances at the onset of the separation. The flow path is the same as that during stop-flow relaxation. When the desired channel and cross flow rates and pressures are achieved,  $V_1$  is switched back to its original position, and the separation and elution process begins.

The valve configuration shown in Figure 2 can be simplified by replacing  $V_1$  and  $V_2$  with a six-port valve.

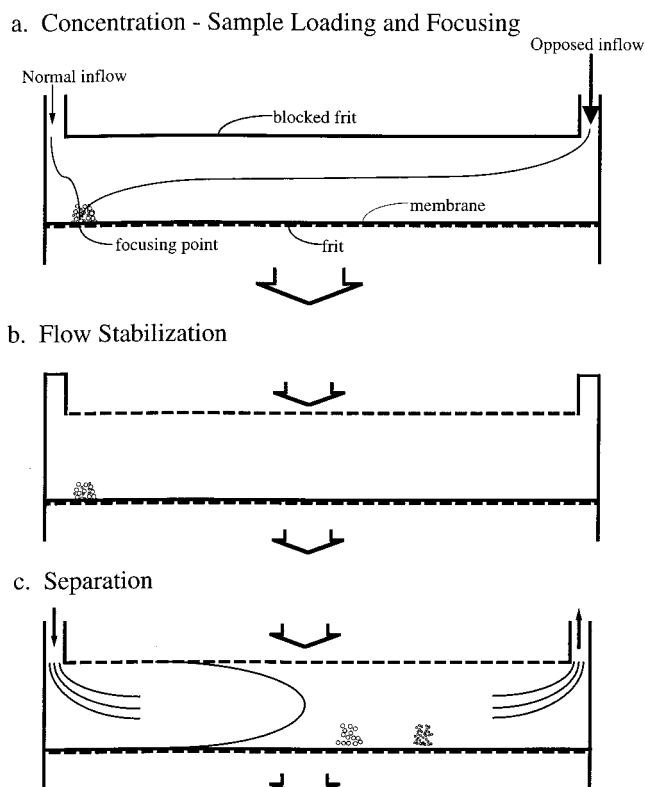


Figure 3. The three stages of the experiment.

**Determining Channel Void Volume.** The flow rate ratio of the two inflows determines the position of the focusing point along the channel length,  $L$ . For instance, a 1:10 ratio of forward:reversed inflow results in a focusing point,  $1/11$  or  $0.09L$  from the normal inflow inlet. The focusing point is usually established downstream from the tip of the FFF channel to avoid hydrodynamic disturbances<sup>25</sup> and sample overloading because of the reduced channel area. The focusing point must be accurately determined and the corrected void volume used to calculate diameter values. The corrected void volume can be calculated from the retention time measured for a PS latex bead of known diameter. Without this correction, the diameter results will be shifted to smaller diameters.

**Measuring Sample Recovery.** Sample recovery is measured by running the sample through the FFF channel under normal separating conditions and by injecting the sample directly into the detector (bypassing the FFF channel). The ratio of the peak area of the FFF run to the channel bypass run multiplied by 100 yields the percent sample recovered.

## RESULTS AND DISCUSSION

**Opposed Flow Sample Concentration of Polystyrene Latex and Protein Standards.** Mixtures of polystyrene (PS) latex beads were initially used to determine the effectiveness of the OFSC technique. Dilute sample suspensions were prepared and introduced into the flow FFF channel as described in the previous section. The flow rate at the normal inflow inlet and the opposed inflow inlet are represented by the symbols  $\dot{V}_F$  and  $\dot{V}_O$ , respec-

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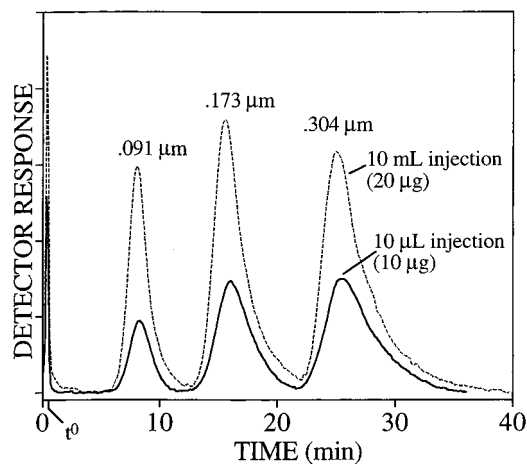


Figure 4. Fractograms of PS latexes obtained by stop-flow and concentration runs. In the first case, 10  $\mu\text{L}$  containing 10  $\mu\text{g}$  of each latex was injected; in the second case, 10 mL containing 20  $\mu\text{g}$  was introduced into the channel. The focusing flow rate is 1:13.2 with flow rates at the normal ( $\dot{V}_F$ ) and opposed ( $\dot{V}_O$ ) inflow inlets of 0.26 and 3.37 mL/min, respectively (corresponding to a focusing point at 1.87 cm from the normal inflow inlet). The concentration time is 50 min. During the separation,  $\dot{V} = 4.31$  mL/min,  $\dot{V}_c = 0.95$  mL/min.

tively. The channel and cross flow rates are denoted as  $\dot{V}$  and  $\dot{V}_c$ , respectively. The fractograms resulting from a 10 mL sample loading with OFSC and a regular 10  $\mu\text{L}$  injection with stop-flow relaxation are superimposed in Figure 4. The retention times and the resolution are comparable for both runs with a maximum difference of  $\pm 3\%$  for the former. The channel efficiencies are also in good agreement. Differences in peak areas between the two runs are a result of different injected sample amounts (10  $\mu\text{g}$  for stop-flow and 20  $\mu\text{g}$  for OFSC). As expected, the peak area for each PS latex standard in the concentration run is approximately twice that of the stop-flow run. These results represent a sample concentration factor of  $10^3$ . Concentration factors as high as  $10^5$  (1 L sample volume) have been achieved, albeit with some loss of resolution.

The OFSC fractogram shown in Figure 4 was obtained by loading the 10 mL sample into the channel through the normal inflow inlet (forward direction). Using the conditions detailed in the caption of Figure 4 and the first term on the right-hand side of eq 1, the sample loading time is 38 min. The sample loading time can be reduced to 3 min by introducing the sample through the opposed inflow inlet. However, the focusing time will be significantly increased since the sample will have a greater distance to travel to reach the focusing point while being entrained in the slow flow region near the membrane wall for a significant fraction of the time. Consequently, sample introduction through the opposed inflow inlet where the flow rates are  $\sim 10$  times faster than at the normal inflow inlet does not necessarily lead to a reduction in the time required for concentration. A theoretical analysis has shown that, for the flow rates listed in Figure 4, sample loading at the opposed inflow inlet would result in a reduced concentration time only if the sample volume exceeded 100 mL.<sup>20</sup>

The reproducibility of the flow FFF separations with OFSC was determined for 10 mL volumes of PS latex beads and proteins. Fractograms of consecutive runs are superimposed in Figure 5, and the results are summarized in Table 1. It should be noted

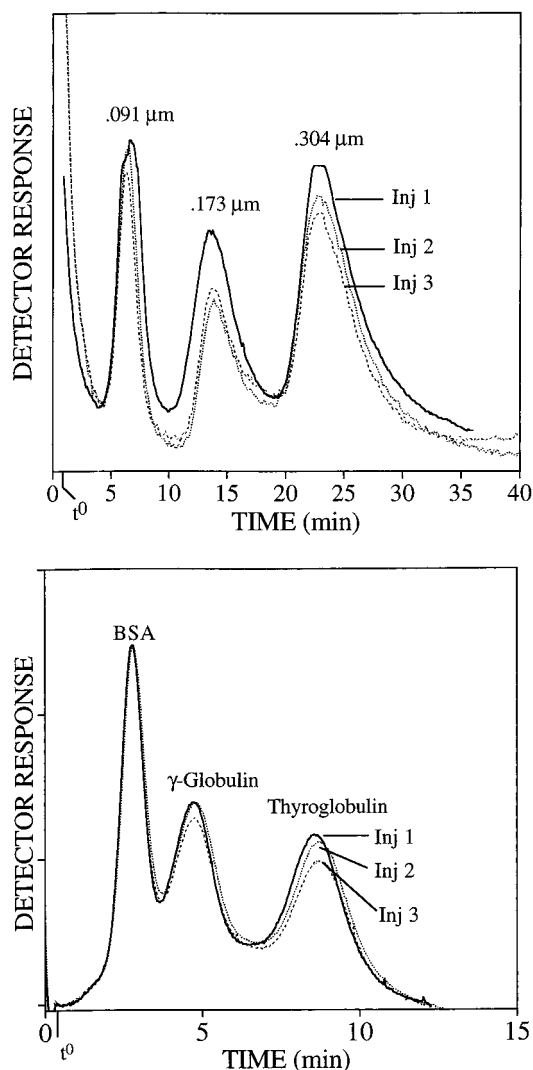


Figure 5. (a) Three consecutive runs of polystyrene latex beads. Injection volumes are 10 mL. The focusing flow rate ratio is 1:13.5 with  $\dot{V}_F = 0.28$  mL/min and  $\dot{V}_O = 3.84$  mL/min. The concentration time is 50 min. During separation,  $\dot{V} = 4.31$  mL/min,  $\dot{V}_c = 0.95$  mL/min. (b) Runs of three proteins. Injection volumes are 10 mL. The focusing flow rate ratio is 1:13.4 with  $\dot{V}_F = 0.26$  mL/min and  $\dot{V}_O = 3.48$  mL/min. The concentration time is 50 min. During separation,  $\dot{V} = 2.71$  mL/min,  $\dot{V}_c = 4.18$  mL/min.

that in both cases, the flow rate conditions employed were not aimed toward achieving high-resolution separations. An increase in cross flow rate and/or decrease in channel flow rate would yield higher resolution. The relative standard deviations (RSD) in retention time ranged from 1 to 3% and are similar to those obtained for regular stop-flow runs with microliter sample volumes. The RSD of the peak area (representative of sample recovery) ranged from 6 to 14%. The error of  $\pm 15\%$  reported for ultrafiltration of trace elements in river waters reflected a comparable relative standard deviation.<sup>26</sup> With rigorous cleaning and sampling procedures, the reproducibility in recovery of dissolved organic carbon in seawater using cross flow ultrafiltration and a concentration factor of 12.8 was of the order of  $\pm 2\%$ .<sup>27</sup> For OFSC of both

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Table 1. Percent Relative Standard Deviations (% RSD) of Retention Times ( $t_r$ ) and Peak Areas of 10 mL OFSC Runs

|                                      | % RSD <sub>tr</sub> | % RSD <sub>area</sub> |
|--------------------------------------|---------------------|-----------------------|
| polystyrene latex beads <sup>a</sup> |                     |                       |
| 0.091 $\mu\text{m}$                  | 0.79                | 8.2                   |
| 0.173 $\mu\text{m}$                  | 2.2                 | 7.8                   |
| 0.304 $\mu\text{m}$                  | 2.6                 | 14                    |
| proteins <sup>b</sup>                |                     |                       |
| bovine serum albumin <sup>b</sup>    | 0.98                | 5.5                   |
| $\gamma$ -globulin                   | 0.64                | 8.5                   |
| thyroglobulin                        | 0.66                | 11.3                  |

<sup>a</sup> Focusing ratio 1:13.5 ( $V_F = 0.28$  mL/min,  $V_O = 3.84$  mL/min);  $\dot{V} = 5.21$  mL/min,  $V_C = 1.27$  mL/min; 0.1% SDS + 0.02% NaN<sub>3</sub>. <sup>b</sup> Focusing ratio 1:13.4 ( $V_F = 0.26$  mL/min,  $V_O = 3.48$  mL/min);  $\dot{V} = 2.71$  mL/min,  $V_C = 4.18$  mL/min; PBS buffer, pH 7.2, 20 mL ethanol rinse between runs.

Table 2. Recovery of Jordan River Colloids after OFSC with Different Membranes Using 0.1% FL-70 and 0.02% NaN<sub>3</sub> Carrier Liquid

| membrane  | peak area (arbitrary units) |
|---|-----------------------------|
| regenerated cellulose (YM-30, Amicon Corp. <sup>a</sup> ) | 740                         |
| polysulfone (PM-30, Amicon Corp. <sup>a</sup> )           | 668                         |
| polypropylene (Celgard 2400, Hoechst Celanese)            | 867                         |
| polycarbonate (30 nm pores, Poretics <sup>b</sup> )       | 7692                        |

<sup>a</sup> Presently part of Millipore Corp. <sup>b</sup> Presently Osmonics.

protein and PS latex samples, the reproducibility in peak area declined with increasing retention time (and thus closer proximity to the membrane surface).

Channel cleaning between runs was essential in obtaining good reproducibility in peak areas. When OFSC was applied to dilute protein solutions, a 25 mL injection of ethanol was used to clean the channel between runs. Reproducibility improved and the channel pressure which was observed to increase after each OFSC run (possibly due to blocked membrane pores) was returned to initial levels. In the case of PS latex beads, this ethanol treatment was more effective than flushing the channel with high flow rates.

**Selection of Membrane and Carrier Liquid for the Analysis of Environmental Particulates.** The membrane accumulation wall is the heart of the flow FFF channel. Desirable membrane properties include smoothness, uniform pore size and distribution, compatibility with the carrier liquid, and minimal interactions with sample species. The last property, measured as sample recovery, was the determining factor in identifying a suitable membrane for river colloid analysis. The recoveries of Jordan River colloids after OFSC and separation by flow FFF are summarized in Table 2. These results are reported as peak areas rather than percent recoveries because the low concentration of river colloids present in the sample yielded no detector signal when a channel bypass run was performed. The ultrafiltration and polypropylene membranes, commonly used in flow FFF, yielded very poor recoveries. An aluminum oxide-coated polypropylene membrane was also evaluated, but was found to lose its coating after 3 runs as evidenced by a visual inspection. The membrane showing the

best recovery was the PVP-coated polycarbonate membrane. The PVP coating renders the polycarbonate membrane hydrophilic, imparts a slightly negative charge, and may prevent sample adsorption through steric hindrance.<sup>28</sup> The PVP is not chemically bonded to the membrane surface and appears to be slowly washed off by the carrier liquid. A continuous decrease in the recovery of the river sediment standard was observed over three consecutive runs. When the membrane was conditioned by injecting 20 mL of 0.1% PVP solution between runs, the RSD in sample recovery decreased from 18% to 8%. This RSD can undoubtedly be further lowered as better membrane cleaning and conditioning procedures are developed.

River sediment and aluminosilicate particle (kaolin) standards were utilized as test samples to determine the carrier liquid that would yield the highest sample recovery. A 0.03  $\mu\text{m}$  polycarbonate membrane formed the accumulation wall of the flow FFF channel. Carrier liquids that were evaluated were doubly distilled deionized water containing (1) 0.1% SDS (anionic surfactant) + 0.1% NaN<sub>3</sub> (bactericide), (2) 0.1% FL-70 (anionic-nonionic surfactant mixture) + 0.02% NaN<sub>3</sub>, (3) 0.05% C<sub>12</sub>H<sub>25</sub>NH<sub>2</sub>HCl, (cationic surfactant), and (4) 0.05% (Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>)·10H<sub>2</sub>O + 0.05% (NaPO<sub>3</sub>)<sub>6</sub> (dispersants for inorganic particulates<sup>29</sup>). Carrier liquids 1 and 2, containing anionic surfactants yielded the highest sample recovery. Carrier liquid 1 was used in all subsequent experiments because SDS consists of a single chemical moiety, whereas FL-70 is a complex mixture of salts, organics, and surfactants. Interpretation of results and trends would be more straightforward with SDS.

Sample recovery is often measured as the ratio of the peak area of a retained peak to the peak area of a channel bypass run. This method of measuring recovery is fast and applicable to most samples. However, misleading results can be obtained when the particulate sample is complex and contains submicrometer and supramicrometer-sized populations. The signals from the UV absorbance detectors that are used in these particulate analyses arise from light scattering and absorption. Since the light scattering signal is a function of particle size, selective loss of large particles through membrane adsorption could distort the recovery results. For example, if supramicrometer-sized particles such as kaolin are selectively adsorbed onto the membrane, their loss could result in a disproportionately large change in peak area and thus give the semblance of a major sample loss. Whether this indeed happens was determined by comparing the ratio of supramicrometer to submicrometer-sized particles in the original river sediment standard to that in the fractions collected after flow FFF. The results using scanning electron microscopy (SEM) yielded a supramicrometer:submicrometer particle ratio of 0.340 for the original sample suspension and 0.366 for the collected effluent. These values, based on counting more than 2000 particles, confirm that no significant size selective adsorption had occurred and that the peak area can be used as a measure of sample recovery.

**Flow FFF Analysis of a River Sediment Standard.** All experiments were done using a flow FFF channel equipped with a 0.03  $\mu\text{m}$  polycarbonate membrane and an aqueous carrier

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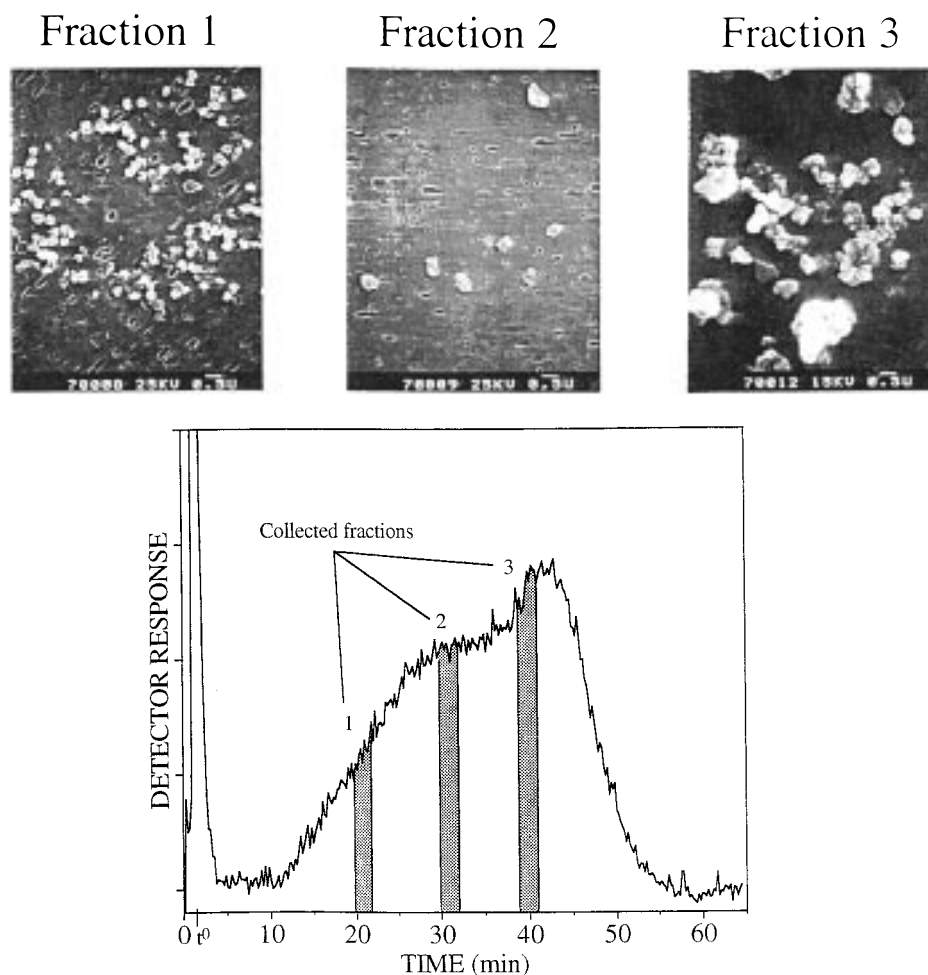


Figure 6. Fractogram and SEM micrograph of river sediment standard. For the run,  $\dot{V} = 0.97$  mL/min,  $\dot{V}_c = 0.18$  mL/min.

containing 0.1% SDS and 0.1%  $\text{NaN}_3$ . (A detector was connected to the cross flow outlet to ensure that no sample was lost through the membrane.) A  $10\ \mu\text{L}$  aliquot of a  $1.7\ \text{mg/mL}$  river sediment suspension was injected into the flow FFF channel where it underwent stop-flow relaxation, separation, and elution from the channel. The resulting fractogram and scanning electron micrographs of fractions that were collected over 2 min intervals are shown in Figure 6. The micrographs confirm that a size fractionation has been achieved with smaller particles eluting prior to large particles. Since flow FFF separations are dependent on only the hydrodynamic diameter,<sup>23</sup> other physicochemical properties of the sample components are inconsequential and particle size information is readily calculated from the retention times. The river sediment possesses a broad continuous distribution of particles ranging in size from  $0.1$  to  $1.2\ \mu\text{m}$ . (The sharp reduction of the detector signal toward the end of the run in Figure 6 is due to steric inversion.<sup>30,31</sup>)

The diameters of particles present in each collected fraction were measured from the micrographs and compared to the diameters calculated from FFF theory based on the time of fraction collection.<sup>22</sup> The SEM results listed in Table 3 were obtained by averaging measurements made along the longest and shortest

Table 3. Comparison of Expected and Actual Diameters of Particles Collected after Separation by Flow FFF

| fraction number   | time of cut (min) | diameter based on time of cut ( $\mu\text{m}$ ) | diameter based on SEM ( $\mu\text{m}$ ) |
|---|-------------------|---|---|
| River Sediment Standard, Stop-Flow Relaxation, $10\ \mu\text{L}$ Sample |                   |   |   |
| 1   | 20–22             | 0.266–0.296                                     | $0.298 \pm 0.021$                       |
| 2   | 30–32             | 0.431–0.469                                     | $0.484 \pm 0.093$                       |
| 3   | 39–41             | 0.633–0.693                                     | $0.636 \pm 0.102$                       |
| Jordan River, OFSC, $10\ \text{mL}$ Sample                              |                   |   |   |
| 1   | 23–25             | 0.231–0.272                                     | $0.185 \pm 0.030$                       |
| 2   | 32–34             | 0.352–0.406                                     | $0.481 \pm 0.113$                       |
| 3   | 44–46             | 0.568–0.650                                     | $0.734 \pm 0.149$                       |

axes of the irregularly shaped particles. Since FFF theory was developed for spherical particles, the good agreement between the predicted and actual diameters indicates that these irregularly shaped river sediment particles are close to spherical. The trend of increasing particle diameter with retention time is clear.

**OFSC Flow FFF Analysis of Particulates in Jordan River Water.** Ten milliliters of Jordan River water was loaded onto a flow FFF channel. The OFSC technique was employed using the conditions indicated in the Figure 7 caption. Sample loading and focusing required a total of 50 min. Fractions were collected and examined by SEM. Although the fractogram is very broad and

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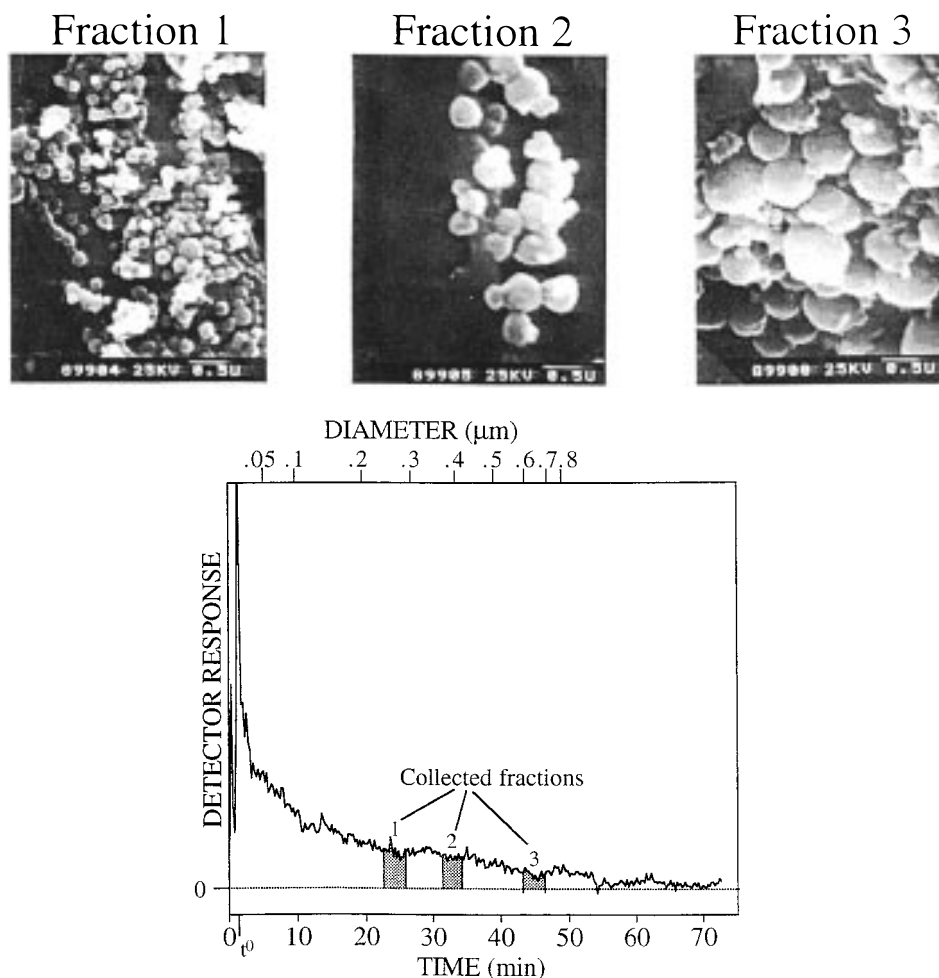


Figure 7. Fractogram and micrographs of Jordan River sample obtained by flow FFF with OFSC. Focusing flow rate ratio is 1:13.7, and the concentration time is 50 min.  $\dot{V}_F = 0.25$  mL/min, and  $\dot{V}_O = 3.43$  mL/min, and during separation  $\dot{V} = 1.07$  mL/min,  $\dot{V}_c = 0.24$  mL/min.

featureless, the electron micrographs show that the OFSC technique was successfully applied to natural waters and that flow FFF was able to separate a continuous distribution of particle sizes. The micrographs show the expected increase in particle diameter with increasing retention time. The clumping of particles in each fractogram is attributed to the SEM sample preparation process.

The predicted and experimental particle diameters in each fraction of the Jordan River sample are summarized in Table 3. For fraction 1, the actual diameter obtained from micrographs is smaller than predicted and the reverse trend is observed for fractions 2 and 3. Energy-dispersive X-ray spectrometry showed that the elemental composition of the particles in fraction 1 was mainly silicon and that in fractions 2 and 3 was mainly aluminum and silicon. Fractions 2 and 3 are probably aluminosilicates such as kaolin. Since kaolin particles are flat, the effective spherical diameter measured by FFF will be smaller than the SEM diameter which is measured across the face of the particle.

## CONCLUSIONS

The application of flow FFF to environmental studies has thus far been retarded by the lack of a membrane with good sample rejection properties. In this paper, we have shown that the recovery of river colloids is 10 times higher when the ultrafiltration membranes commonly used in flow FFF are replaced by a PVP-

coated polycarbonate membrane. We have also observed that membrane cleaning and conditioning between runs can lead to significant improvements in reproducibility.

The opposed flow sample concentration method has been shown to be equally applicable to the on-line concentration of dilute sample suspensions of proteins and particles. Subsequent separation by flow FFF and examination of fractions collected at the channel outlet by scanning electron microscopy confirmed that a size fractionation has been achieved and that the concentration process had no adverse effects on the separation. Since the entire process of sample loading, concentrating, and separating is implemented by a series of switching valves, OFSC is readily automated.

With the development of the opposed flow sample concentration method, two-dimensional FFF may now be realized. Other FFF techniques, such as sedimentation, electrical, and thermal FFF, could be directly coupled to flow FFF and information about particle density, mass, shape, and surface charge and composition could be correlated with size. Alternately, other types of FFF channels could be modified with a permeable frit wall segment to allow OFSC to be performed. In the area of environmental studies, OFSC provides a simplified alternative to off-line concentration procedures. In addition, OFSC can be applied to different volumes of river water to gain insight into the problem of artifact



formation that has been reported for samples prepared by ultrafiltration.

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