



Microscale Purification Systems for Biological Sample Preparation

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Abstract. This work is focused on the development of miniaturized instrumentation formats for integrated biochemical and biological sample preparation. In particular, the paper is focused on microfluidic systems for the purification of cells, uncoated nano particles, and surface coated nano particles. Microfluidic systems will be described for purifying cells from whole blood, sorting of blood cells based on their electrophysiological characteristics, and separating and purifying the components of a blood cell homogenate. Additionally, the microscale sample preparation systems will be shown to be effective in purifying complex samples of nano particles based on the particles physical size and effective electrical charge. The microsystems will be demonstrated for the purification of samples containing polystyrene nano particles with multiple diameters as well as for samples containing a mixture of uncoated and surface coated nano particles. The formats discussed in this work include the micro-electrophysiological characterization (μ -EPC) system; the micro-thermal field flow fractionation (μ -TFFF) system; and the micro-electrical field flow fractionation (μ -EFFF) system. The microscale cellular electrophysiological characterization system is used for cell sorting and selection. The cell sorting and selection is accomplished using impedance spectroscopy on single cells. The system has been shown to be effective in sorting blood cells by cell type (i.e., red, white) and by sub populations (e.g., viable leukocytes, non viable leukocytes). The μ -TFFF system is a chromatographic separation technique for fractionating particle samples based on the heat capacity, thermal conductivity and physical size of the particles. The μ -EFFF system fractionates particle samples based on the physical size and zeta potential (effective electrical charge) of the particles within the sample. The electrical and thermal FFF systems are capable of separating particle samples with constituents in the diameter range from approximately 1 nm to 1 μ m. The separation and purification of a variety of nano particles will be demonstrated in the field flow fractionation systems including cells, cellular sub-components, uncoated polystyrene nano particles, and protein coated (Protein A) polystyrene nano particles.

Key Words. micro-analysis, sample preparation, micromachining

Introduction

The miniaturization of biochemical analysis systems has been a topic of growing interest over the past decade. During this time, a large majority of the technical effort

has been invested in the development of the primary separation (or amplification) component of the various analysis systems such as the microcolumns used in the miniaturized chromatographic systems (e.g., electrophoresis, gas chromatography, liquid chromatography) or the chambers used for miniaturized polymerase chain reaction (PCR) systems. Less effort has been directed toward the development of technologies for microscale sample preparation (with the exception of PCR). Sample preparation technologies include methods for purifying, manipulating, interfacing, amplifying, and chemically modifying sub-microliter volumes of samples for analysis in a miniaturized format. While each of these technologies is available in a macro scale format, most have not been available on the microscale until recently. During the past two to three years, there has been a significant increase in the worldwide efforts to improve the technology base for miniaturized sample preparation. These efforts have resulted in microsystems for purifying and sorting biological samples, manipulating samples, sample amplification, and interfacing samples with microanalysis systems [1].

In this paper, we will focus on the use of micromachining technologies for the development of three different microsystems for use in integrated sample preparation. The three systems include: (a) the micro-electrical field flow fractionation system; (b) the micro-thermal field flow fractionation system; and (c) the micro-electrophysiological characterization system.

Micro-Electrophysiological Characterization (μ -EPC)

Electric impedance (EI) measured in a 1-dimensional electric field is an established method for interrogating the electromagnetic behavior of isolated materials and

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composite systems. Microfabrication techniques offer a low-power means of applying traditional EI concepts to the investigation of complex-valued dielectric properties of small structures and material samples. Reducing the overall size of an EI measurement device allows for increased spatial resolution while limiting the possibility of dielectric breakdown by minimizing the strength of the required electromagnetic field. In addition, microscale EI devices require only nanoliters of sample solution.

Microfabricated EI sensors have demonstrated the ability to recognize variations in solution temperatures, ionic concentrations, hydrogen peroxide concentrations, and even antigen-antibody binding (immuno-sensors) [2,3]. Previously reported systems interrogate solutions using an array of surface mounted metal electrodes with an active surface area of $\sim 1 \text{ mm}^2$. To further reduce the required sample size, we fabricated EI systems containing microchannels lined with metal electrodes. The devices allow materials to be positioned between interrogating electrodes using rapid and efficient fluid transport methods [4–6]. Although microscale EI devices may have numerous applications in basic science and engineering, their size makes them especially suitable for biological studies.

Investigations into the dielectric properties of proteins are one area of possible applications for an integrated microchannel/EI measurement system. The electrical characteristics of protein solutions are believed to play an important role in their physiologic functions involving protein-protein and charged ligand interactions [7–9]. In these studies, the dielectric dispersion of protein solutions is attributed to a frequency dependence on permittivity and attempts were made to relate the observed electrical characteristics to the summation of distributed charges within the protein (polarization vector) and proton fluctuations.

An additional biological application for a microscale EI device is the measurement and extraction of the dielectric properties of single living cells. Numerous techniques have been developed to extract the canonical parameters (i.e., membrane capacitance, membrane resistance, and cytoplasmic resistance) of both individual cells and cell aggregates. The three most common techniques include the EI cell suspension technique [10–12], whole-cell patch clamping [13–15], and electrophoresis/electrorotation [16–27]. All of these methods rely on estimates of cell geometry for the accurate calculation of the electrical parameters and are generally limited to whole-cell resolution. Microchannels outfitted with EI measurement electrodes may provide increased spatial resolution (by using electrodes smaller than a single cell) and known cellular geometry (by constraining the cell within the microchannel) [28].

The microsystem is suitable for impedance measurements of femto liters of fluids, solutions, suspended particles, and single cells within microchannels. Figure 1 is a schematic of the microsystem in which two fluid reservoirs are connected by a single microchannel which narrows to a width of $\sim 10 \mu\text{m}$ (channels are $\sim 4 \mu\text{m}$ high). The microchannels were constructed from epoxy-based photoresist on quartz glass wafers. Full-depth gold measurement electrodes were integrated into the narrowest portion of the microchannels. Through holes were wet etched in microscope coverslips and bonded to the microstructures to form the top surface of the microchannels. The resulting microchannels and electrodes are sandwiched between planar glass substrates exhibiting excellent optical qualities and allowing for direct sample observation using transmission light microscopy. Parasitic capacitance between the electrodes is minimized by isolating the electrodes on all sides with materials with high dielectric constants (glass and epoxy based photoresist). The μ -EPC measurement system was characterized using biological concentrations of ionic salt solutions, air, and partially de-ionized (DI) water over the frequency range of 100 Hz to 2 MHz [29].

Figure 2 is a scanning electron micrograph of a microchannel with a bonded coverslip overlaid on an image of an electrode/microchannel device prior to bonding. The narrow region of the microchannel in the figure measures $\sim 10 \mu\text{m}$ wide and $4.3 \mu\text{m}$ deep with the gold electrodes measuring $8.0 \mu\text{m}$ wide and $\sim 4.0 \mu\text{m}$ thick. The gold electrodes typically extended into the channel 1 to $2 \mu\text{m}$ on each side resulting in an average electrode gap of $7.13 \mu\text{m}$. Prior to coverslip bonding, the depth of the channels was measured using a Dektak IIA

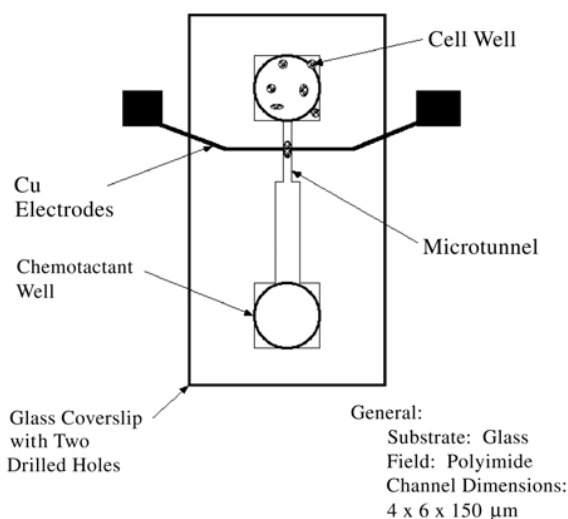


Fig. 1. Schematic view of the micro-electrophysiological characterization system.

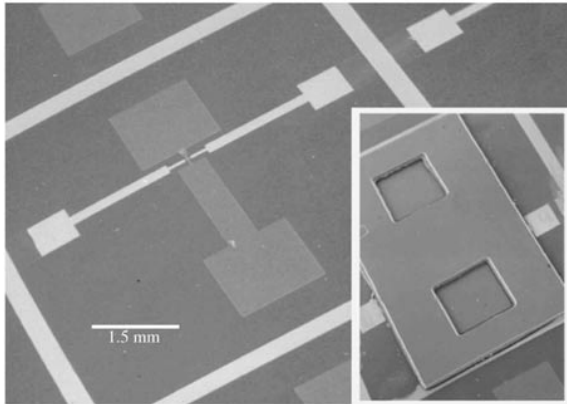


Fig. 2. A SEM image of a coverslips bonded to the bottom substrate can be seen overlaid on an image of a electrode/microchannel device prior to bonding.

profilometer (Sloan Technologies, Santa Barbara, CA) and found to vary by only 0.11 μm with a mean value of 4.31 μm . Devices with a total of eight different microchannel and electrode geometries were designed and fabricated on each wafer. Square holes were wet etched completely through the 200 μm thick coverslip glass in addition to etching each coverslip free from the adjacent neighbors. The gold bonding pads are shown in Figure 2 extending from the edges of the bonded glass coverslip.

The $\mu\text{-EPC}$ was used to measure the total electrical impedance (EI; designated Z) between the gold micro-electrodes over a frequency range of 100 Hz to 2 MHz.

The impedance is a function of the geometry and properties of the device as well as the interaction with the cell/media in the recording zone. To characterize the device and the electrode-electrolyte interface, we measured Z after filling with several isotropic materials having known dielectric properties. Example results are provided in Figure 3 (A,B) for air (thick solid curves), partially deionized water (dot-dashed curves) and several concentrations of phosphate buffer solution (PBS) as indicated in the figure legend. The equipment did not allow for clean records above 100 MOhm. It is notable that the microsystem behaves nearly as an ideal capacitor when filled with air, as expected, but has a much more complex behavior when filled with physiological concentrations of PBS. Characteristics of the metal-electrolyte interface are primary determinants underlying the PBS impedance curves.

We have also measured electrical impedance between the microelectrodes when individual polymorphonuclear leukocytes (PMNs) and individual red blood cells (RBCs) were positioned directly between the electrodes. Example results are shown in Figure 3 (C,D) for PMNs ($n = 28$) and in (E,F) for RBCs ($n = 50$). Both the magnitude and phase were highly repeatable between different cells. Results for PMNs were very interesting in that two distinct subpopulations were easily identified on the basis of the EI data. Averages for type I cells are shown in thick dashed lines and averages for type II cells are shown as thick solid lines in Figure 3 (C,D). Type I cells correspond to non-viable PMNs and type II cells correspond to viable, healthy PMNs. It is well known that the permeability of

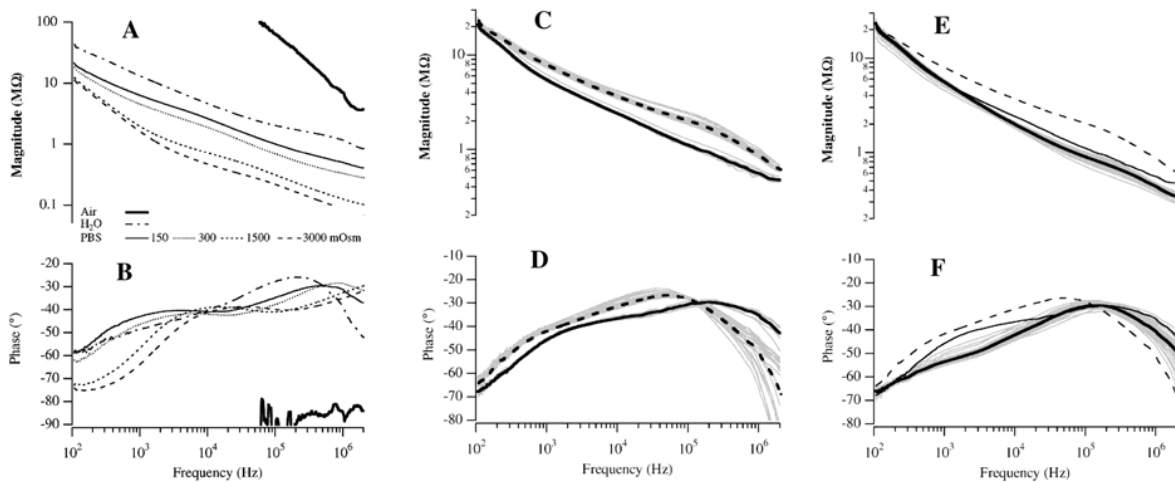


Fig. 3. The electrical impedance was measured across the gold microelectrics using a HP4194 Impedance Analyzer. Curves on the left (A,B) were recorded when the channel was filled with air, partially DI water and concentrations of PBS. Curves in the center (C,D) are for the same $\mu\text{-EPC}$ when PMNs were positioned directly between the electrodes. Gray solid curves indicate individual cells, the black dashed curves show the average for PMN type I cells, and the black solid curves show the average for PMN type II cells. Results for RBCs are shown on the far right (E,F). Thick black solid curves are reproduced from C and D for comparison.

cell membranes decrease upon cell death. Also, notable is the difference in the data between the PMNs and the RBCs. Clearly, the μ -EPC was capable of distinguishing between the two cell types as well as between viable and non-viable PMNs. In either case, these preliminary studies indicate potential for the μ -EPC in cell sorting and characterization. It is also notable that the largest intercellular differences were recorded at the highest frequency tested, 2 MHz. This is expected due to the dielectric constant of the membrane allowing a stronger trans-membrane electric field at high frequencies. Differences between cells are most easily seen in the phase for PMNs, Figure 3D, which may reflect differences in cytoarchitecture between the recording electrodes.

Micromachined Thermal Field-Flow Fractionation (μ -TFFF)

TFFF is an elution separation technique similar to chromatography except the separation field is normal to the sample and carrier flow. TFFF utilizes thermal diffusion as the separation field instead of the gel, liquid, or column packing found in chromatographic separations. This field is accomplished by a temperature gradient across the channel. A schematic of the TFFF system is shown in Figure 4. Separation of the suspended particles is performed in a solvent carrier such as methanol, tetrahydrofuran, acetonitrile, or dimethylsulfoxide. Many solvents have been used and there have been studies showing how these solvents affect separation characteristics [30]. Water is not typically used as a carrier fluid unless an electrolyte has been added. The particles in the solvent react to the temperature gradient by diffusing toward the cold wall. Higher molecular weight particles react more to the thermal gradient and are compacted more tightly against the cold wall than do lower molecular weight particles. Because of the laminar velocity profile of the carrier, samples that compact less will have a higher average velocity than the samples that compact more. The difference in average velocity results in the spatial and temporal particle separation at the output of the TFFF channel [31].

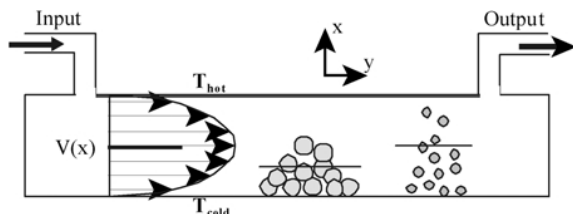


Fig. 4. Schematic of a thermal field-flow fractionation (TFFF) channel.

The TFFF system has some unique characteristics making it more suitable for some separations than conventional systems. In TFFF, the separation field is applied normal to the separation. The resolution requirement in the direction of separation is no longer needed, which means lower field strength, lower power consumption demands, and shorter separation times. TFFF also has the advantage of elution techniques, in that the samples are collected in fractions at given times. As a result, very pure samples can be obtained [31].

In order for a separation in a TFFF channel to occur, there must be:

1. A difference in molecular weight or diameter of the samples;
2. A sample selective perturbation of the samples toward one wall; and
3. A laminar velocity profile that results in a different average velocity of each sample constituent [32].

The channel height is a critical parameter is the spatial resolution of two eluting samples. The channel height is inversely proportional to the resolution. Current systems have channel widths of about 127 μm [32]. Micromachining technologies allow accurate and precise definition of channels of very small size. The channels fabricated for the μ -TFFF device are approximately 25 μm . As a result, higher resolution separations can be performed which means faster separations and more pure samples.

The μ -TFFF system was fabricated with an n-type, (100), single side polished, silicon wafer using common micromachining processes. A silicon dioxide (SiO_2) mask was thermally grown at 1200°C to approximately 1 μm thick on the surface. Photolithographic techniques were used to define the input/output ports in the photoresist on the backside of the wafer. The SiO_2 mask was etched using a buffered hydrofluoric acid solution. The ports were then anisotropically etched through the silicon wafer in 10% potassium hydroxide (KOH) at 90°C.

The SiO_2 was left on the wafer as an electrical insulating layer for the heaters. Titanium (Ti) was sputtered onto the backside of the wafer to about 1000 Å and patterned to define the resistive heater.

A negative photosensitive epoxy (SU-8) was spun on the wafer to a thickness of 25 μm . The channel was defined in the SU-8 by UV exposure and developing. Heat treatment of the channel walls ensured a strong bond and durability when exposed to the carrier fluid, which is typically an organic solvent. A glass microscope slide was adhered to the top of the SU-8 to complete the channel. After completing the fabrication of the μ -TFFF separation channel, the complete system was assembled (e.g., fluid interconnections, power supply, flow meters, detector, and fraction collector) and tested.

Using the μ -TFFF device fabricated as described in this paper, the total plate height was determined as a function

of flow velocity for an unretained sample. DI water was used as the carrier and the sample was pure acetone. The water flow rate was set by the pump. The flow rates used were 2.0, 1.75, 1.5, 1.25, and 1.0 mL/hr. An acetone sample, 0.2 μ L, was injected into the input port at time zero for each flow rate. A detector measured the absorbance with respect to a sample of DI water.

The retention of polystyrene (PS) spheres was then tested in the system, since separation is based on the difference in retention for different sized particles. Although water is not typically used for TFFF separations because of the difficulty in performing separations in this medium, DI water was used to demonstrate the ability of the μ -TFFF system. The flow rate was set to 1.5 mL/hr to minimize the plate height. A temperature gradient of 40°C ($T_{\text{hot wall}} = 70^\circ\text{C}$, $T_{\text{cold wall}} = 30^\circ\text{C}$) was set up using the integrated heater and an external heat sink. A 0.2 μ L sample of PS spheres (394 nm) was injected into the system.

One end of a fabricated channel and port is shown in Figure 5. The channel height has been reduced from 127 μm (macro-TFFF system) [7] to 50 μm (μ -TFFF). A power of only 10 W was required to achieve a 20°C temperature difference across the channel. This power is more than 1000 times lower than that reported for a typical macro-scale TFFF channel. Plate height was determined as a function of average carrier velocity and compared with results from the μ -EFFF system [33] and TFFF theoretical results. The plate height curve is shown in Figure 6. The μ -TFFF plate height characteristics follow the curves that are found in the μ -EFFF channel as well as the theoretical curve for the TFFF system. The data are of lower magnitude than the sum of plate heights reported for retained polystyrene samples (MW_n : 154 k, 392 k, and 735 k). The temperature gradient was 30°C and toluene is the carrier. The channel dimension is 0.025 cm \times 1.2 cm \times 305 cm [34].

Retention and fractionation of a sample with PS-154 and PS-394 is shown in Figure 7. Three peaks appeared as a result of the test. The first peak is the void peak

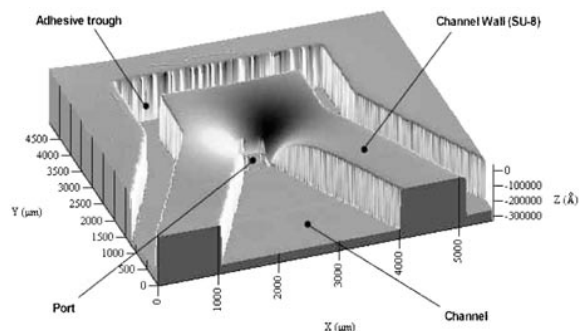


Fig. 5. Profile of one end of the μ -TFFF channel.

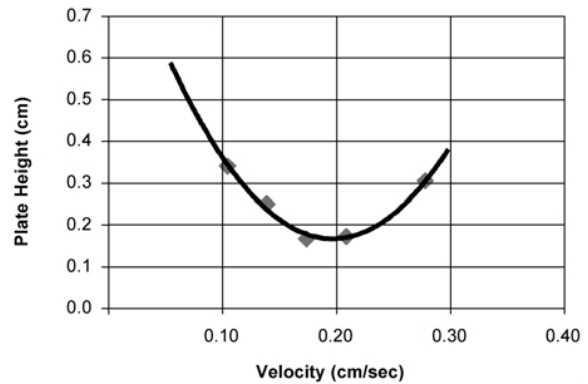


Fig. 6. Plate height as a function of flow velocity. This characteristic is typical of field flow fractionation systems.

which contains unretained impurities. The second peak eluted at 590 seconds and was the 154 nm polystyrene particles. The third peak eluted at 635 seconds and was the 394 nm polystyrene particles. This result indicated the effectiveness of the μ -TFFF system in purifying samples in preparation for further processing.

Micromachined Electrical Field-Flow Fractionation

Another system with significant potential in the area of sample preparation is the micromachined electrical field-flow fractionation (μ -EFFF) system. Electrical field-flow fractionation is a particle separation technique that relies on an electric field perpendicular to the direction of flow and separation as shown in Figure 8. The separations are performed in a low-viscosity liquid (typically an aqueous buffer solution) which is pumped through the separation channel. EFFF controls the relative velocity of particles by forcing particles towards the wall of the channel.

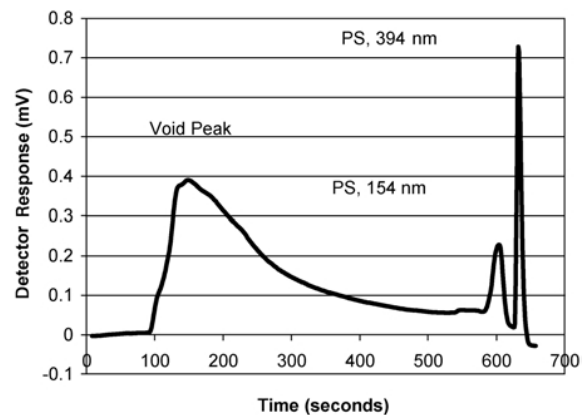


Fig. 7. Retention and fractionation of PS-154 nm and PS-394 nm spheres.

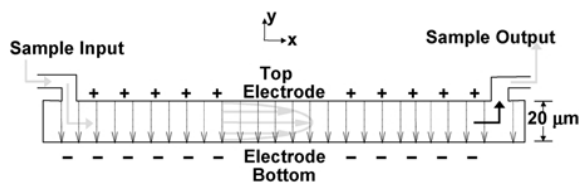


Fig. 8. Schematic diagram of the operating principle for the EFFF system.

Particles with high charge density pack closer to the wall and move more slowly compared to particles of lower charge density that form a more diffuse cloud and move more quickly through the channel.

The channels for the miniaturized EFFF system are shown schematically in Figure 9 and are fabricated by bonding a silicon substrate and a glass substrate together around a photolithographically defined polyimide spacer. Both substrates have electron beam deposited metal deposited and patterned to define the driving electrodes and the sensing electrodes for the electrical impedance detector. The input and output ports are fabricated in the silicon substrate using KOH etching and are 200 μm square on the channel surface. The channel dimensions are typically about 6 cm long, 1–6 mm wide, and 10–50 μm in height. Fabrication and other information about the system have been reported previously [35]. The system also has an integrated electrical impedance detector that has been incorporated at the exit end of the channel [1,36,37].

One of the main advantages associated with EFFF is that it is an elution method that allows for the collection of fractions at the exit to the system. Thus, EFFF, which separates by particle size and charge, can produce a monodisperse sample for later analysis using another system. EFFF has an additional advantage in that it is a very gentle separation method and is suitable for cells,

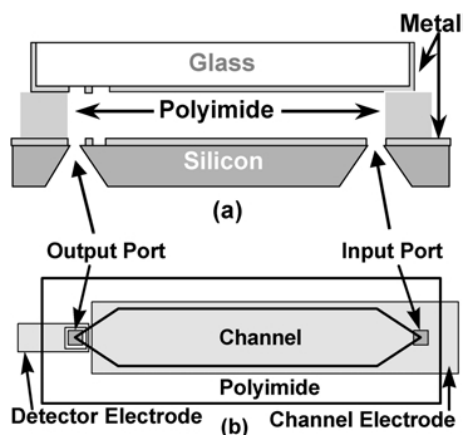


Fig. 9. Schematic of channel and detector layout (a) Side view of channel (b) Top view of channel.

liposomes, micelles, fragile proteins, and other delicate structures and polymers. Particles ranging in size from about 5 nm up to about 1 μm in diameter are separable using a micromachined EFFF (μ -EFFF) system. Combining EFFF on a chip with other analysis systems would provide the opportunity for performance of a sample purification or separation step using EFFF and then, by simply redirecting the flow at appropriate intervals, allowing the analysis to continue in an orthogonal direction, using another system, or in a parallel direction using a similar system.

The μ -EFFF system has been demonstrated with a range of polymers as well as a few biological samples of interest. A typical separation is shown in Figure 10. In this figure, a high-speed separation of a three-component mixture of polystyrene samples was accomplished. As demonstrated here, EFFF separates particles of similar material based entirely on the particle size. Thus a polydisperse sample would show up as a wide peak on the x -axis with each slice in time or volume representing a monodisperse sample. Accordingly, collection of a small volume of sample at a given location provides a well-defined, monodisperse sample for further analysis.

The micromachined EFFF system also has the ability to separate identically sized particles based entirely on electrophoretic mobility. In this case, the particles of lower charge will elute before particles with a higher charge as shown in Figure 11. The μ -EFFF would then also be available to prepare samples that differ only by surface charge characteristics.

A biologically valuable separation of this type was demonstrated in the μ -EFFF system when particles with adsorbed proteins were differentially retained from those without any adsorbed protein. The results of such an

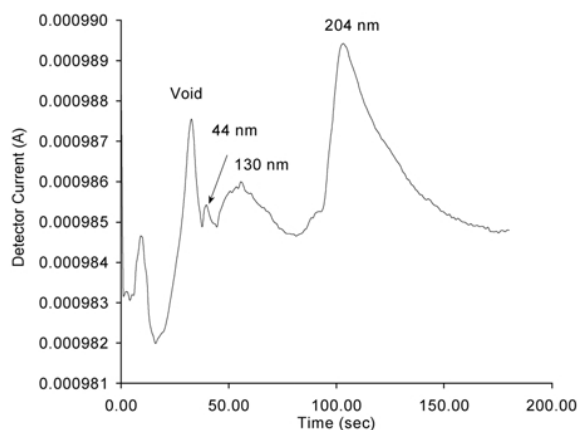


Fig. 10. Separations of 44, 130, and 204 nm polystyrene particles with on-chip AC mode detector showing steadily increasing diameter of eluting particles. Voltage was 1.6 V, flow rate was 0.3 mL/hr and current was 27 μA .

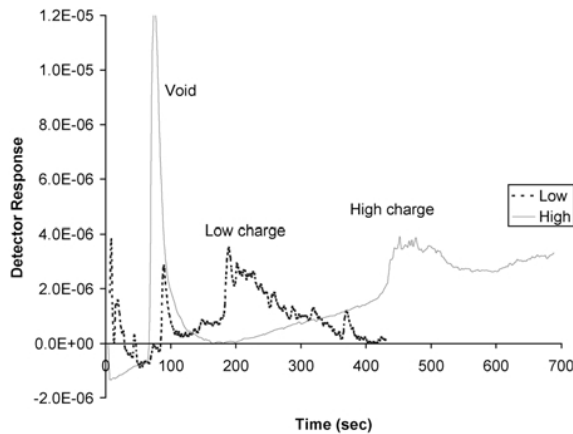


Fig. 11. Fractograms of particles with the same diameter, but differing levels of carboxylation. The run labeled “low” had a lower density of COOH groups on the surface and a correspondingly lower surface charge, so the particles eluted sooner than did those with a greater surface charge, labeled by “high.”

analysis are presented in Figure 12. An analysis of this type is very useful in biocompatibility and protein adsorption studies. Since particles with and without protein are retained differentially, particles with adsorbed proteins could be collected for later analysis, assuming they were the particles of interest. In addition, the wide peak for the particles with attached proteins indicates that there is a variation across the peak that corresponds to the amount of protein adsorbed to the particle. Thus, if particles were required to have a minimum level of protein attached to the particle, particles towards the end of the elution peak could be collected, or if a uniform number of attached proteins was required, a thin slice of the peak could be collected to create a monodisperse sample for later analysis.

EFFF also has the ability to retain a wide variety of particles contained in a single sample, such as a blood

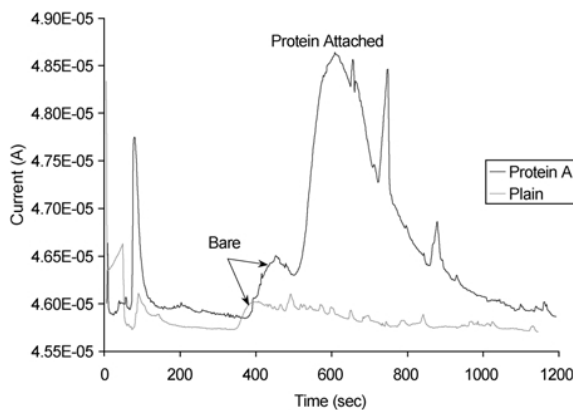


Fig. 12. Fractograms of showing differential retention between bare particles and particles with attached proteins.

sample. Blood analysis is one of the most common medical procedures and the retention of a blood sample is demonstrated in Figure 13. While the cells found in blood are much too large to be significantly retained in a μ -EFFF system, the proteins and other particles found in blood are quite easily retained. Thus, the cells could be separated from the smaller particles, or the smaller particles can be collected for later analysis. The same blood sample is shown for two different runs. In the first run, the retention of the whole blood is shown. The second run shows a sample that was homogenized in an ultrasonic bath. The homogenized sample shows the retention of the cell contents that were not present in the whole blood sample. In both cases, after the elution time is determined for the particle type of interest a fraction of that sample could be collected for later analysis. Thus, μ -EFFF demonstrates its ability to function as a sample preparation system for a wide range of analytes and particles of interest.

Conclusion

In this work, three microsystems are discussed for miniaturized biochemical sample preparation. The micro-electrophysiological characterization system was demonstrated for use in cell sorting, purification and concentration of cell types. Using the micro-EPC, viable PMN’s, non-viable PMN’s and red blood cells were separated and sorted from a complex media, blood. The micro-electrical and -thermal field flow fractionation systems were demonstrated as micro device formats for continuous flow systems used to separate and purify samples containing particles, cells and cellular compo-

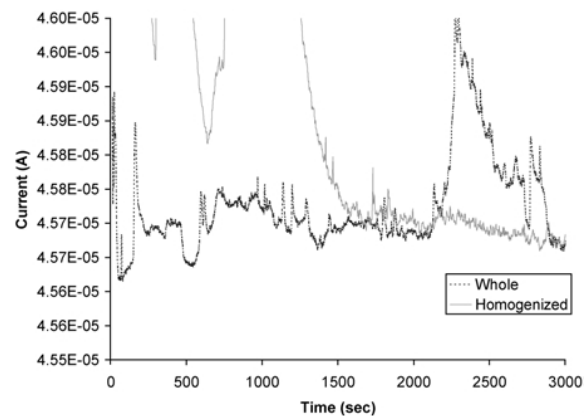


Fig. 13. Comparison of whole blood and homogenized blood retained in the μ -EFFF system focusing on the whole blood fractogram. Note, there is continual elution of particles during most of the run, but a strong peak appears near the end of the run in a range where no elution in the homogenized sample occurred.

nents. These systems, when used in series with each other and complimentary microanalysis systems, offer to potential to realize a total analysis system capable of sample in/answer out operation.

Acknowledgments

Support of this work by the following sponsors is greatly appreciated: National Institute of Environmental Health Sciences ES-10846, Genicon Sciences Inc., MicroChem Corp.

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