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Lab-on-a-chip for drug development

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Abstract

Significant advances have been made in the development of micro-scale technologies for biomedical and drug discovery applications. The first generation of microfluidics-based analytical devices have been designed and are already functional. Microfluidic devices offer unique advantages in sample handling, reagent mixing, separation, and detection. We introduce and review microfluidic concepts, microconstruction techniques, and methods such as flow-injection analysis, electrokinesis, and cell manipulation. Advances in micro-device technology for proteomics, sample preconditioning, immunoassays, electrospray ionization mass spectrometry, and polymerase chain reaction are also reviewed.

Keywords: Microfluidic; Lab-on-a-chip; MicroTAS; Electrokinetic

Contents

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1. Introduction to micro-scale phenomena

Microfluidic devices are often described as miniature versions of their macro-scale counterparts. While this analogy is true for some aspects of microfluidic devices, many phenomena do not simply scale linearly from large to small implementations. Examples include the following: increased surface area-to-volume ratio and the omnipresence of laminar flow.

The first commercial microfluidic lab-on-a-chip-based systems were introduced for life science applications less than three years ago. Since that time the field has also seen the formation of a number of diverse microfluidics companies; the publication of more than two thousand scientific papers on microfluidics; and development of several additional microfluidics-based products. MIT Technology Review named microfluidics one of ten technologies that will change the world and one of the many areas in which this will be seen is in the life sciences sector.

To date, several lab-on-a-chip companies, including Aclara, Caliper, and Orchid Biosciences, have developed microfluidic technologies that work for highly predictable and homogeneous samples that are common in the drug discovery process, whether in compound screening, genomic analysis or proteomics [1–5]. The first generations of many of these systems address the non-FDA-regulated life sciences research market. One of the primary challenges for homogenous sample-based lab chip providers, however, is their inability to perform analysis on chip directly from normal, complex and heterogeneous clinical samples, such as whole blood.

Other companies active in the microfluidics area (e.g., Micronics, Cepheid, Fluidigm, and Nanostream) have tried to address this issue by their choice of chip materials, structure, and dimensions, by selecting a fluid transport method that is compatible with biological fluids, and by constructively allowing for the handling of blood and cell-laden streams on microchips.

In 2001, Kricka [1] surveyed the range of micro analytical devices, from microchips and gene chips to bioelectronic chips, and their impact on diagnostic testing. He predicted a move of clinical testing from central laboratory to non-laboratory settings with a positive impact on healthcare costs.

1.1. Characterization of fluid flow

Fluid flow is typically characterized as one of two regimes: laminar or turbulent. These two different regimes are characterized by the relative importance of inertial to viscous forces, which is usually described by the Reynolds number [2–4], the ratio of inertial and viscous forces on fluid flowing in a channel, i.e. a ratio of the momentum of the fluid to the friction force imparted by the channel walls. Originally proposed by Osborne Reynolds in 1883 [5], it describes the character of the flow.

A low Reynolds-number flow is a laminar, or layered, flow in which fluid streams flow parallel to each other and mix only through convective and

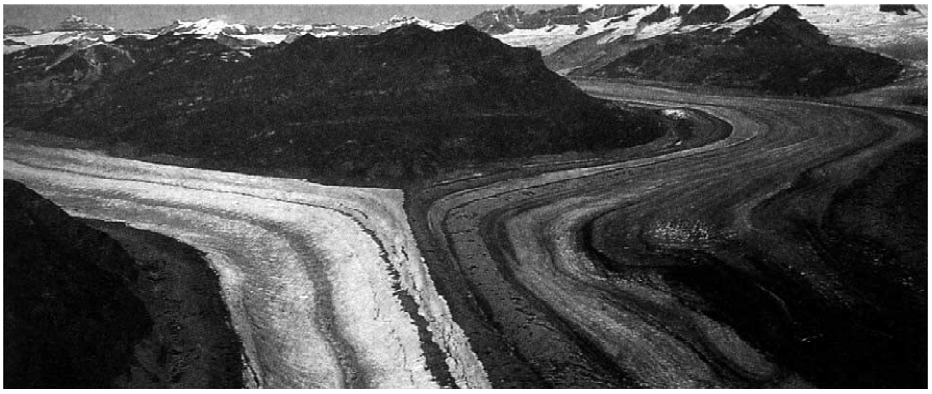


Fig. 1. A glacier illustrates laminar flow. No mixing occurs between the two side-by-side streams of ice.

molecular diffusion (see Fig. 1). Laminar flow is dominated by viscous forces; fluid velocity at all locations is invariant with time when boundary conditions are constant. There is convective mass transport only in the direction of fluid flow. A high Reynolds-number flow is a turbulent flow in which inertial forces dominate and various-size parcels of fluid exhibit motions that are simultaneously random in both space and time. Significant convective mass transport occurs in all directions. The transition between laminar and turbulent flow typically occurs above $\mathrm{Re} = 2000$ in internal flows. The Reynolds number is defined as

$$\mathrm{Re} = \frac{v\, D_H}{\mu} \tag{1}$$

where v is the average velocity of the fluid in the channel, μ is the viscosity of the fluid, and the hydraulic diameter D_H is ''four times the cross-sectional area divided by the wetted perimeter'', which leads to

$$D_H = \begin{cases} d & \text{pipe diameter} \\ 2h & \text{slot height} \\ \dfrac{2}{1/h + 1/w} & \text{channel height and width} \end{cases} \tag{2}$$

depending on the type of channel, e.g. round pipe, wide slot with height/width ratio less than 0.1, or channel with rectangular cross-section.

1.2. Why use microfluidic technology?

Microfluidic devices present unique advantages for sample handling, reagent mixing, separation, and detection. This technology is ideal for handling costly and difficult-to-obtain samples and reagents. Typical microfluidic structures require between 100 nl and 10 μl of sample and reagents.

Microfluidic channel dimensions typically range from 1 to 1000 μm in width and height. Frequently, only one channel dimension is within that range; this is sufficient to provide laminar flow characteristics following Eqs. (1) and (2). In addition to obvious advantages that are associated with smaller sample, reagent, and waste volumes, several phenomena occur differently at these dimensions than in larger-scale traditional devices:

- All flow in such channels tends to be laminar, thus allowing the parallel flow of several layers of fluid. This enables the design of separation and detection devices based on laminar fluid diffusion interfaces, which will be discussed later.
- At these dimensions, diffusion becomes a viable method to move particles, mix fluids, and control reaction rates. For example, Cephradine (MW 349), a small antibiotic, diffuses about 14.3 mm/s at room temperature in aqueous solutions. This allows the establishment of controlled concentration gradients in flowing systems, as well as complete equilibration of the

molecule across a 100 μm channel in less than 1 min without any active mixing elements.
- Sedimentation at standard gravity, unaided by centrifugation, becomes a viable means to separate dispersed particles by density across small channel dimensions. For example, cells in slightly-diluted whole blood sediment in a 100 μm deep channel in about 1 min, and generate a 50 μm layer of slightly-diluted plasma in the process.
- Active particle transportation and separation methods, such as capillary electrophoresis, show greatly enhanced separation performance in small channels.
- In microfluidic channels, the diffusion distance can be made extremely small, particularly if fluid streams are hydrodynamically focused. Thus diffusion-controlled chemical reactions occur more rapidly than in comparable macroscopic reaction vessels. For example, Hatch et al. [6] have shown a microfluidic immunoassay that was completed in less than 25 s, as opposed to more typical immunoassay reaction times of 10 min or more.

In addition to these unique phenomena, there are additional reasons to pursue micro-scale technology:

- Plastic microfluidic structures can be mass-produced at very low unit cost, allowing them to be made disposable.
- Micro devices are amenable to high throughput by processing several assays in parallel.
- Micro devices require only small volumes of sample and reagents, and produce only small amounts of waste, which can often be contained within the disposable device.
- The micro scale allows development of total-analysis systems (Micro-TAS) capable of handling all steps of the analysis on-chip, from sampling, sample processing, separation and detection steps to waste handling. This integration makes complex analyses simpler to perform.

As might be expected in a field less than 30 years old, significant technical challenges remain. These include, but certainly are not limited to, the following issues:

- Interfaces between the macroscopic and microscopic environments. This area includes sample introduction and extraction as well as sample interrogation.
- Mixing of fluids without turbulence in a timely fashion.
- Fluid transport. Two main methods are currently in use (see following section) electroosmotic pumping and pressure-driven flow. Each method has advantages and disadvantages; neither one provides a generic solution to the fluid transport problem.

2. Methods of moving fluid in microfluidic devices

One of the most critical elements of any microfluidic system is its fluidic transport system. Depending on the size of the fluidic channel network, such fluid drivers need to be capable of generating flow rates ranging from hundreds of microliters per minute for high-volume-throughput applications such as flow cytometry [7] down to picoliters per second for applications using micron or submicron-sized channels [8].

Numerous different fluid driving methods for microfluidics have been reported. Electroosmotic, gas-pressure, positive displacement, micro-peristaltic, thermal, and many other pumping modes, or combinations thereof, all have their advantages and disadvantages. However, the two fluid driving modes most commonly used are electroosmosis and hydraulic pressure, and they will therefore be discussed in more detail in the following subsections.

2.1. Pressure-driven flow

One of the more common methods of generating pressure for fluid flow in microfluidic circuits is positive displacement pumping [9]. The main advantage of positive displacement is that such circuits have very little compliance, which makes controlling the exact amount of pumped fluid, and knowing the exact location of a fluid meniscus much easier. Some

disadvantages are mechanical complexity, size, and frequently the fact that no such pumps are commercially available for lower flow rates. Ultra-precise syringe pumps often are used for this purpose [10–13]. Caliper Technologies has developed a platform that provides both electrokinetic and pressure-driven fluid flow [14]. Frequently, however, even the most precise commercially available syringe pumps do not provide a flow that is smooth enough for many microfluidic applications. Micronics (Redmond, WA), in conjunction with Honeywell (Minneapolis, MN) has developed a flow-sensor-calibrated ultra-precise syringe pump unit as used by Jandik et al. [15].

An additional complication is that pressure-driven flow in a channel exhibits a non-uniform velocity profile, which is pseudo-parabolic (depending on the aspect ratio of the channel), maximal in the center of the channel and decreasing to zero velocity immediately proximal to the channel walls. The fluid dynamics of this phenomenon are well-understood and are modeled by the Navier–Stokes equations with proper boundary conditions [4,3]. The implications of this behavior for microfluidic assays remain an area of significant research [13,16].

2.2. Electroosmotic flow

A second method of moving fluids in microfluidic devices takes advantage of the well-known phenomenon of electroosmotic flow (EOF). EOF occurs in devices with channel walls made of materials that are charged under experimental conditions. The fluid comprising the double-layer proximal to the charged surface will not be neutral, but rather will contain a higher-than-bulk fluid concentration of counter-ions, resulting in a charged fluid (see Fig. 2). If an electric potential is applied parallel to the channel walls, this charged fluid will be attracted to the electrode of opposite charge, resulting in convective fluid flow with a blunt (uniform) velocity profile [17]. The bulk velocity v can be calculate using the following equation from Sherbet [18]:

$$v = \frac{\zeta \epsilon_r \Phi}{4\pi \mu}$$

where v is the average velocity of the fluid in the channel, ζ is the zeta potential at the channel wall, ϵ_r is the dielectric constant of the fluid, Φ is the electric field strength, and μ is the viscosity of the fluid.

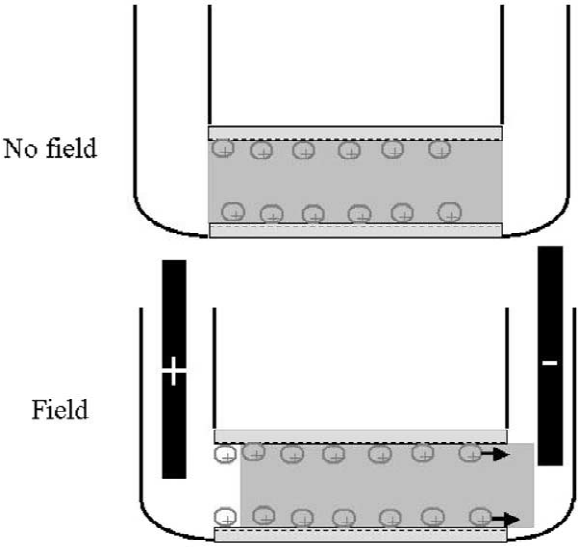


Fig. 2. Schematic of electroosmotic flow.

EOF-driven flow is clearly dependent on the interaction between the channel wall surfaces and the enclosed fluid. EOF-driven flow is particularly efficient in channels of less than 0.1 mm in diameter [19]. EOF-driven flow has been successfully implemented with a range of channel materials, most commonly glass [20], although EOF has also been used in polymeric devices [21,22]. Capillary electrophoresis devices are driven almost exclusively by EOF [20]. Glass devices with EOF-driven flow have been used successfully to mix different solutions, perform chemical reactions, and immunological assays [23]. Various groups have experimented with different surface modifications to change the wall surface charge, and therefore the velocity of the fluid undergoing EOF, via either covalent modification of the surface or through additives in the fluid [24–27]. The electroosmotic mobility (EOM) of polydimethylsiloxane (PDMS) and its ability to support electroosmotic flow were investigated [28]. EOM was increased four-fold in low ionic-strength solutions at neutral pH in oxidized PDMS channels, but high ionic-strength buffers at neutral pH compatible with living eukaryotic cells greatly diminished this effect.

Bruin [29] reviewed microfluidic separation devices in which the flow is electrokinetically driven. Examples were given of microchip layouts, functional elements, use of alternative materials to glass, and multiple detection methods. Bousse [30] discussed applications of electrokinetically-driven microdevices, including: DNA separations, enzyme assays, immunoassays, and polymerase chain reaction (PCR) amplification integrated with microfluidic assays.

2.2.1. Electrokinetic flow control

An electroosmotically-induced hydraulic micropump was developed that consists of a tee-junction with one inlet held at high voltage and one outlet at ground [31]. The remaining outlet has no electric potential and experiences pressure-induced flow when electroosmosis in the ground channel is reduced by a viscous polymer coating. Anions can be switched between outlets by changing the flow resistance in the field-free channel relative to the ground channel. In another electroosmotically-pumped device gaseous electrolysis products were removed though a permeable polymeric membrane [32]. The electroosmotically-induced fluid velocities were independent of the field strength and length of the electroosmotically-pumped region, but dependent on applied voltage.

Field-effect control was achieved on a microfluidic system fabricated from PDMS on a silicon wafer base that was electrically-grounded and had an electrically-insulating layer of silicon dioxide [33]. Varying the polarity and the magnitude of the resulting radial electric potential gradient across the silicon dioxide layer gave direct control of the zeta potential and the resulting electroosmotic flow.

2.2.2. Electrokinetic mixing

Because microfluidic flows are typically laminar, complete mixing cannot be achieved by the typical macro-scale techniques. Techniques that employ micro-scale physics are being investigated. A 100 picoliter static mixer using multiple intersecting channels and driven by electroosmotic flow was microfabricated, tested, and theoretically simulated [34]. The extent of mixing was determined by confocal microscopy and CCD detection. Another microdevice, a T-microchannel imprinted in polycarbonate and laser-modified to create a series of slanted wells at the junction, was reported to mix two streams within 0.44 mm instead of 23.0 mm as required using diffusion only [35]. An alternate strategy to intersecting channels is the use of sinusoidally-oscillating electroosmotic channel flows [36]. The optimal mixing technique for each application depends on the particular microdevice, but the fundamental physical basis for mixing in the microscale is diffusion.

2.3. Summary of fluid handling

EOF provides a blunt flow profile but is very sensitive to variations in both fluid composition and channel wall coatings, thus limiting its use as a generic pumping solution. Pressure-driven flow is significantly less sensitive to such variations (fluid viscosity being a notable exception) but results in a non-uniform flow profile, which is rarely a benefit. The optimal choice of fluid transport system remains application-specific.

3. Micro-construction techniques

There are many different ways to manufacture microfluidic devices. Kovacs [37] provides a good review of traditional lithographic methods.

3.1. Traditional lithographic techniques

Micro-device construction techniques began as a offshoot of the computer chip processing industry, in which hard substrates, most commonly silicon, are used as the primary construction material. The basic paradigm of standard lithographic techniques involves the use of electromagnetic radiation, typically ultraviolet (UV) light, to transfer a pattern to a surface, such as silicon, covered with photoresist. The ‘‘mask’’ that contains the pattern can be as simple as an overhead with the desired design printed on it or more elaborate, such as a chrome mask. The pattern is transferred to the photoresist-covered surface by shining electromagnetic radiation through the mask onto the surface. Typically ultraviolet (UV) light is used, although electromagnetic

waves with narrower wavelengths, such as X-rays, have been used to achieve a finer resolution. The photoresist is then developed such that the areas exposed to the electromagnetic radiation behave in an opposite manner to unexposed areas; one set of areas polymerizes and remains on the surface while the other set is washed away. Now that the mask has been transferred to the surface, any one or a number of surface modification techniques, or a sequence of techniques, can be deployed. Chemical etchants, in which exposed surfaces are ''eaten away'' while protected surfaces remain, are one example of surface modification techniques. Glass and silicon are the two most typical substrates used for fabricating microfluidic devices with these methods. A recent review by McCreedy [17] offers a detailed summary of the majority of these techniques.

Many variations on these techniques have been reported. A lateral percolation filter was created by deep reactive-ion etching 1.5×10 micron channels in the bottom of quartz microchannels [38]. Flow through the filter was driven by electroosmosis triggered by silanol groups on the walls and was free of particles of sizes from dust to cells. Manufacture of a microfabricated electrophoretic device with 40 cm long channels was announced [39] for human genome sequencing. It achieves an average of 800 base reads compared to 550 with capillary array electrophoresis. A microfabricated nozzle with a volume of less than 25 picoliters was developed for electrospray of liquids for analysis by mass spectrometry [40]. It showed up to three times more sensitivity and approximately half the signal variation compared to a pulled capillary. A glass-based multireflection absorbance cell for microchip-based capillary electrophoresis was photolithographically fabricated with aluminum mirrors positioned above and below the flow channel [41]. The device demonstrated higher absorbance sensitivity than previous planar devices. Powderblasting was presented as a technique for direct etching of microchannels [42]. A universal conductivity detector thus fabricated was able to detect micromolar concentrations of charged species. Conductivity detectors have been reported to have a lower relative standard deviation than the current monitoring method for measuring electroosmotic flow [43]. In addition, electrolyte conductivity is linear with electrolyte concentration and could be used to measure extent of mixing in electrokinetically-driven flows.

These methods are amenable to parallel processing and can produce mechanically strong and chemically-resistant devices, with feature sizes as small as 0.1 m. However, these methods are also expensive, require significant chemical processing equipment outlays, and often require toxic chemicals. In addition, the processes are often time-consuming and not amenable to rapid design iterations. Newer methods of MEMS fabrication address many of these concerns.

3.2. Soft lithography

Recent developments in MEMS fabrication techniques have moved away from the mask/etch paradigm inherited from the computer microprocessor industry and instead exploit the properties of polymers. One common method is to use traditional lithographic techniques to create a template in a thick layer of photoresist, (see Fig. 3). This template can then be filled with a heat- or UV-curable polymer and used to cast numerous replicas of the desired device. Dr. George Whitesides' group at MIT [44–46] and Dr. David Beebe's group at University of Wisconsin [47] have developed many soft lithographic techniques using polydimethylsiloxane (PDMS) and UV-curable polymers. Some examples of the use of these soft lithographic methods are described below.

Fabrication with hydrogels facilitates building device components that respond to chemical factors, such as pH, in the local environment [48,49]. One group has created pH-sensitive values that direct fluid flow depending on the pH of the fluid [50]. Two imprinting techniques were used to fabricate microfluidic devices on poly methyl methacrylate (PMMA) substrates: creating an impression with a wire in heated substrate and imprinting with a mold micromachined on a silicon waver [51]. Reproducible electrophoretic injections and an immunoassay were demonstrated. Analysis of DNA fragments by another wire-imprinted PMMA device was demonstrated [52].

Methods are described for fabrication of free-standing, non-cylindrical three-dimensional microstructures from two-dimensional patterns [53]. Mi-

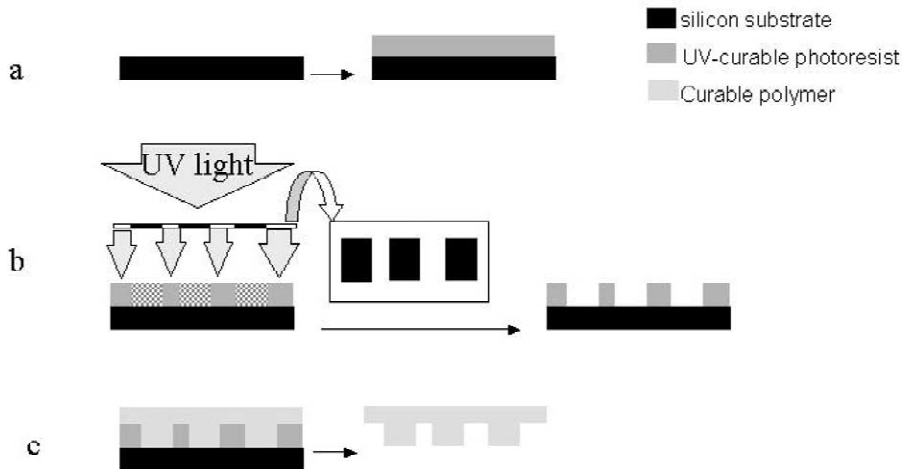


Fig. 3. One example of soft lithography. (a) Using silicon as substrate, generate layer of UV-curable photoresist; (b) use mask to selectively develop sections of photoresist, rinse away undeveloped photoresist to create template; (c) pour curable (heat, UV, chemical) polymer over template, cure, and remove from template.

croelectrodeposition adds strength to the thin metal designs. Another method described as the ''membrane sandwich'' was developed to produce topologically complex three-dimensional microfluidic channel systems in PDMS [44]. An example created was a square coiled channel that surrounds, but does not connect to, a straight channel.

In 2000, Becker [54] reviewed a wide variety of microfabrication methods for polymer substrates, including: hot embossing, injection molding and casting, molding-master fabrication, laser ablation, layering techniques, layer bonding, and dicing.

Fabrication of microfluidic channels with polyelectrolyte multilayers to alter surface charge to control flow direction was reported [55,56]. Flow in opposite directions in the same channel was achieved by using oppositely-charged polyelectrolytes on opposite walls and measured by fluorescence imaging and particle velocimetry.

PDMS-glass microchips with eight parallel capillary-electrophoresis channels were designed with cross and tee injectors that have narrow sample channels instead of uniform channel width and depth at the injection intersection [57]. Improved resolution, column efficiency, and sensitivity were demonstrated.

PDMS is an excellent material for construction of microfluidic networks, but its hydrophobicity results in uncontrolled adsorption on channel walls. A three-layer biotin–neutravidin surface coating was developed that decreases adsorption without inhibiting electroosmotic flow [58]. The modified surface can be patterned with biochemical probes to enable affinity binding assays.

3.3. Laminate technologies

Laminate fabrication methods are based on a different paradigm than either of the two methodologies described above. As with the other technologies, device design begins with designing patterns. However, in laminate fabrication the device consists of layers of material, lamina, that have been laser-cut or stamp-cut into the desired shape and then held together with some form of adhesive, most commonly pressure-sensitive or thermally-activated adhesive. Mylar is presently the most commonly-used material, although other materials such as glass and PDMS have also been successfully incorporated into laminate devices. Laminate fabrication has been used to build devices ranging in complexity from a simple single-component module [59,60] to complicated fluidic circuits used to perform hematology analyses.

Micronics' microfluidic circuits [61] comprise

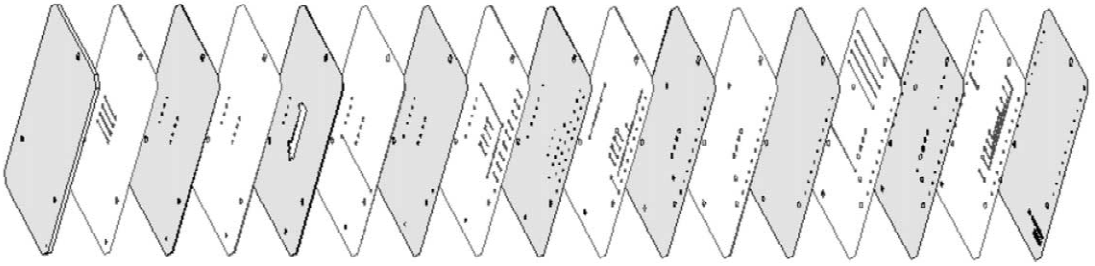


Fig. 4. A blow-up view showing the individual layers that are stacked up to form a laminate card.

laminates built of several layers of individually cut or stamped fluidic circuits. While each layer can be manufactured very easily and inexpensively, the lamination process yields complex 3-dimensional microfluidic structures. This allows the design, for example, of 3D hydrodynamic focusing channels for cell analysis, or of multiple separate circuits with crossing channels on a single card. Figs. 4 and 5 show an example of a Micronics disposable.

While other processes such as hot embossing, micro-injection molding, and, in particular, silicon or glass lithographic techniques yield significantly better dimensional tolerances [9], the plastic-laminate method has its major advantages in turn-around time, cost, and the ease of generating 3-dimensional structures, as well as incorporating hybrid elements into the design (such as electrodes, filter membranes, sensors, etc.).

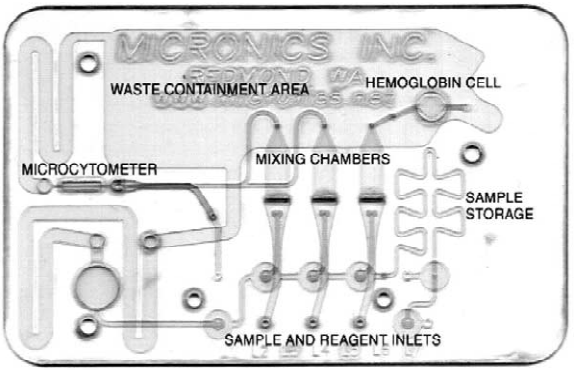


Fig. 5. Micronics disposables are typically credit-card-sized, and most structural elements on these cards have dimensions ranging from about 100 μm to a few millimeters using a low-cost rapid prototyping process that allows the design and testing of new microfluidic structures in 24 h or less.

4. Applications of microfluidic devices

4.1. Laminar fluid diffusion interface

One of the great advantages of microfluidic flow is that it allows the flow and interdiffusion of multiple streams in parallel in a single channel. This method has been discussed in the literature as Laminar Fluid Diffusion Interface (LFDI) technology [9,10,13,6,62]. This method relies heavily on controlled and reproducible introduction of several fluids into one channel, and generally requires either positive displacement pumping or pressurized flow coupled with a feedback flow sensor to provide the desired flow velocity and rate. Other pumping methods tend to be unreliable for this purpose, especially when samples having variable viscosity or ionic background are used.

The LFDI enables diffusion-based separation and detection, which allows the parallel flow of two or more individual streams in a single microfluidic channel. Under microfluidic conditions, fluids usually flow in a very predictable, laminar fashion, thereby allowing miscible fluids (e.g., whole blood and phosphate buffered saline) to flow next to each other without turbulent mixing or the need for physical separation by a membrane. Diffusion naturally occurs under these conditions. Smaller particles (such as ions, small proteins, many drug molecules, etc.) tend to diffuse quickly across the boundary

layer, whereas large molecules and particles, such as cells, tend to diffuse only minimally.

The LFDI effect can be used to both separate particles by size, and to extract components from samples. This principle has been shown, for example, with the H-Filter™ platform (see Fig. 6), which allows a serum equivalent to be generated directly from whole blood without prior use of a filter or centrifuge for sample preparation or provides a desalting step as part of DNA sample preparation. The result is reduction in reagent usage, equipment costs and time.

Fig. 6. Micronics liquid–liquid extraction platform, which uses laminar fluid diffusion interfaces to separate particles by size.

The LFDI effect has been used by Kenis et al. [63] to form channel walls within microstructures by precipitation at the fluid interface.

Micronics' T-Sensor® technology (see Fig. 7) is based on a related principle, and allows the sample molecules to diffuse into a parallel stream containing a reagent and/or an indicator, thus allowing qualitative and quantitative detection. A T-Sensor lab card permits the parallel flow of small quantities of both a sample, a reagent, and a control solution simultaneously in one microfluidic channel. These three flowing solutions naturally form diffusion interaction zones between them. Typically, a fluorescent or absorption indicator may be added to the reagent. The sample or control analyte molecules then diffuse into the reagent stream, where they react with the indicator and form detectable diffusion interaction zones. These zones can be either visually interpreted (i.e., by comparison of width and color to a chart—similar to a test strip) for qualitative or semi-quantitative assays, or they may be monitored with optical systems such as CCD cameras, linear diode

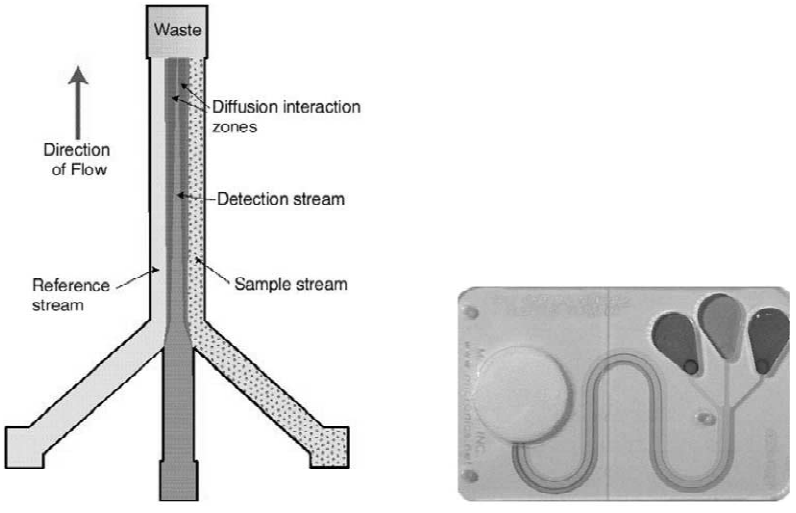


Fig. 7. T-Sensor schematic and a physical realization that is absorption-driven.

arrays, or scanning lasers for quantitative results. By determining the ratio of an optical property of these two zones (using chemiluminescence, fluorescence, absorption), an essentially calibration-free concentration measurement can be derived. With T-Sensor devices, and using standard chemistries developed for serum analysis, the quantitation can take place directly in whole blood—omitting time-consuming centrifugation steps. T-Sensor devices have been used for numerous types of assays including kinetic, protein, enzyme, immunoassays, and electrolyte assays.

Some of the advantages of T-Sensors include:

- Analyte concentrations can be determined optically in turbid and strongly colored solutions, such as blood, without the need for preprocessing such as blood cell removal by centrifugation.
- Many standard optically based (fluorescent or absorption) and electrochemical analytical methods may be performed within the T-Sensor.
- In many cases, indicator-dye cross-sensitivity to larger sample particulates can be avoided in the T-Sensor by making use of the size-based diffusion separation feature of T-Sensors.
- In a T-Sensor, the indicator dye and/or the detection chemistry can be kept in a solution formulated to display optimal characteristics of the detection reaction without affecting the chemical composition and equilibrium in the sample solution. For example, cross-sensitivities to pH or ionic strength can be suppressed by using strongly buffered solutions.
- The location of the diffusion boundaries yields information about flow speed, sample viscosity, sample concentration, dye diffusivity, analyte diffusivity, and dye dynamic range.
- The steady-state nature of this method makes long signal integration times possible. The interface zone of the reagent and sample in the detection window is continuously renewed as the fluids flow past the detector, while the zone that is imaged remains visually the same. This can provide for higher sensitivities than in comparable flow detection systems that rely on the injection of sample plugs into a carrier stream such as Flow Injection Analysis [64], as well as electroosmotically injected sample plugs [30]. For example, a 10 times longer detection time would enable the detector to capture 10 times more photons, and to increase the sensitivity of the assay accordingly.
- In a self-referencing T-Sensor, a control or reference solution containing a known or constant concentration of sample analyte can be analyzed in parallel within the same channel as the sample. This allows real-time referencing and control determination. Therefore, it is possible to compensate for effects such as variations in flow cell geometry, temperature dependent reaction kinetics, light source stability, instabilities in the optical system and detection electronics, as well as fluid parameters such as turbidity, color, concentration of detection chemistry, viscosity, and flow speed.
- Measuring the concentration of the analyte at several locations along the channel may provide an additional means to compensate for residual cross-sensitivities.
- Monitoring signal intensities along the T-Sensor detection channel (in flow direction) provides a means for looking at the kinetics of a reaction, thus allowing kinetic diagnostic reactions to be measured not as a function of time but of distance from the starting point of the diffusion interaction.
- It is possible to flow more than three separate streams through a single T-Sensor, allowing, for example, real-time determination of a sample, as well as a low and high control.
- It is possible to determine two or more different sample analyte concentrations in one single T-Sensor, allowing, for example, the determination of ionized calcium in whole blood while simultaneously compensating for variations in the pH of the blood sample, which affects ionized calcium readings.
- T-Sensor manufacturing is not limited to silicon micromachining. Several other methods have been demonstrated that yield low-cost mass-manufacturable microstructures with similar sizes and geometries.

A critical requirement for achieving a micro total analytical system for the analysis of cells and their

constituent proteins is to integrate the lysis and fractionation steps on-chip [65] for combined cell lysing and enzymatic detection in an LFDI device that combines an H-Filter and a T-Sensor. An integrated microfluidic system for the continuous lysis of bacterial cells and the extraction of β-galactosidase with detection by a fluorogenic enzyme assay was demonstrated. Such a continuous flow device would be a valuable upstream component of further separations by isoelectric focusing or various chromatographic techniques, as well as for analytical techniques such as immunoassays. Here, a microfluidic system integrating the lysis of bacterial cells and the extraction of a large intracellular enzyme, β-galactosidase, was demonstrated.

4.2. Flow injection analysis

One of the earliest inspirations for Labs-on-a-Chip were the integrated Flow Injection Analysis circuits (see Fig. 8) described by Ruzicka et al. [64]. While fluid volumes and circuit dimensions were somewhat bigger than modern microfluidic circuits (mostly because of the size of active elements such as valves), and the fact that the circuits were not microfabricated but machined out of plastic blocks, they still showed all the elements typical for microfluidic analysis systems. More recently, Ruzicka et al. and others [66–68] have shown microfabricated versions of FIA systems. Here, sample plugs are injected into a carrier stream and transported past a detector, thus allowing the rapid manipulation and sequential detection of a large number of samples in a single integrated analyzer.

4.3. Cell manipulation and sensing

Both human cells and bacteria have been successfully transported in microfluidic systems, using either pressure-driven [69,70,60,71] or EOF-driven flow [72]. Bacteria were concentrated using either ZE or IEF [60]. As early as 1987, a silicone microfluidic device was used to transport and fuse individual cells [73]. One group used EOF-driven in combination with a flow injection-type channel assembly to select either bacteria or mammalian cells and then bring the chosen cells in contact with a lysing agent, which would permit downstream analysis of cell contents [72]. More recently, pressure-driven flow was used in conjunction with a micromachined silicon/glass device to transport and position mammalian embryos [71] for processing in preparation for further cell manipulations, such as cell fusion and/or assisted reproduction [74]. Mobilization of biological cells in a microfluidic system by electroosmotic and/or electrophoretic pumping was demonstrated, including steps for cell selection and subsequent reaction [72].

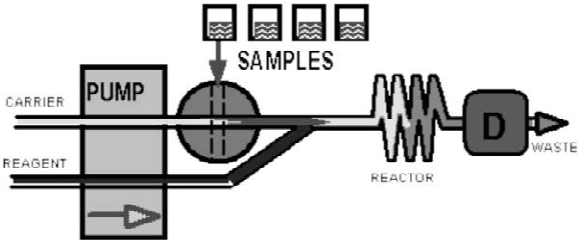


Fig. 8. Schematic of flow injection analysis, FIA.

4.3.1. Flow cytometry

Flow cytometry is based on focusing of cells into a single line for individual analysis. Altendorf et al. demonstrate a microfluidic disposable capable of performing a three-part white blood cell differential [7]. Another device integrates upstream chemical cell-treatment with downstream cytometric detection [61].

PMMA and polycarbonate microfluidic devices with electroosmotic flow and a confocal epi-illumination system were used to detect single labeled DNA (double-stranded) molecules [75]. Excited by a 7 micron Gaussian-profile laser beam, higher sampling efficiency was obtained by narrowing the sampling channel from 50 to 10 microns than by hydrodynamic focusing with sheath electrokinetically-driven from side channels. Molecule sizing using photon-burst detection was inconclusive due to the Gaussian profile of the excitation beam.

An electrophoretically-driven flow microcytometer using coincident light scattering and fluorescence detection was developed [76]. Labeled Escherichia coli were counted at 30 to 85 Hz.

4.3.2. Living cells on self-assembled layers

Techniques for immobilizing viable cells in patterns on microchannel walls have been developed.

Miniaturized sensing techniques for detecting the response of cells has been demonstrated. For example, bacterial antibiotic sensitivity has been detected in cultured cells by microphysiometry based on a light-addressable potentiometric sensor [77,78]. To monitor individual cells surface-creation techniques were developed to position cells accurately inside a microfluidic device. Stable nanometer-scale structures joined by non-covalent bonds were produced by molecular self-assembly [79]. Precisely engineered surfaces for self-assembly were obtained through materials science nanotechnology to obtain optimized reactions with proteins and cells [80]. Condensation figures, the patterns resulting from condensation of vapor on an incompletely-wetted surface, are a nondestructive method for assessing the heterogeneities of the surface [81].

Microcontact printing was demonstrated to be a flexible method to position cells. Cells placed in micrometer-scale arrays by imprinting gold surfaces with an elastomeric stamp [82] provide control over cell growth and protein secretion. Gold structures with edge resolution of less than 100 nm were obtained by lithography of self-assembled monolayers (SAMs) of alkanethiolates by beams of metastable argon atoms [83]. Sub-micrometer patterns have been produced on curved substrates [84] and other topologically-complex surfaces [85] by microcontact printing of SAMs. SAMs of alkanethiolates along with microcontact printing and micromolding in capillaries were used to prepare tissue culture substrates in which both topology and molecular structure of the interface were controlled [86,87]. Patterned SAMs were also demonstrated on optically transparent films of gold or silver on which endothelial cells were then attached and visualized by inverted and/or fluorescence microscopy [88].

The dependence of cell function on cell shape was shown by restricting endothelial cells to progressively smaller micropatterned islands generated by microcontact printing of SAMs of alkanethiolates on gold [89]. A size-related transition from growth to apoptosis was revealed. A follow-on study [90] showed that intermediate-size islands, too small for cell growth and too large to cause apoptosis, caused endothelial cells to differentiate forming capillary tube-like structures containing a central lumen.

Martinoia [91] established patterned populations of neurons on silicon microchips using hydraulically-driven deposition of adhesion molecules. Patterned biological neural networks were formed with chick embryo spinal-cord neurons. Takayama [92] used laminar flow in capillaries to pattern both cell deposition and the media to which they are exposed. They demonstrated patterning of the substrate with different proteins, patterning of adjacent areas with different types of cells, patterning of the chemicals delivered to the cells, and enzymatic reactions over selected cells or portions of a cell.

Microcontact printing and self-assembly have also been used to create a pattern of lines as narrow as 5 microns of self-assembled streptavidin beads on the internal walls of a microchannel [93]. Single beads were precisely positioned and able to withstand forces applied by flowing water in the channel.

4.4. Electrokinetic techniques

One can differentiate between molecules/particles on the basis of many properties, such as density or color, and use such differences to separate, identify, and concentrate these molecules/particles. One such property, surface charge, forms the basis for many well-developed sample handling and analytical methods, referred to as ''electrokinetic'' methods. Examples of electrokinetic techniques include electrophoresis and isoelectric focusing (IEF). IEF and electrophoresis are the two techniques that comprise two-dimensional (2-D) gel analysis, which has become a major tool in proteome analysis, (see Fig. 9). A related technique, dielectrophoresis, relies on the dielectric properties of the particle(s) under investigation.

4.4.1. Electrophoresis

Electrophoresis is a ubiquitous technique in medical analyses, commonly used to fractionate and analyze both DNA and proteins. In recent years its implementation has moved to smaller and smaller scales, from gels to capillaries to microchannels. Electrophoresis is simply the movement of a charged particle in an electric field and has been used for two related but distinct approaches. Particles can be

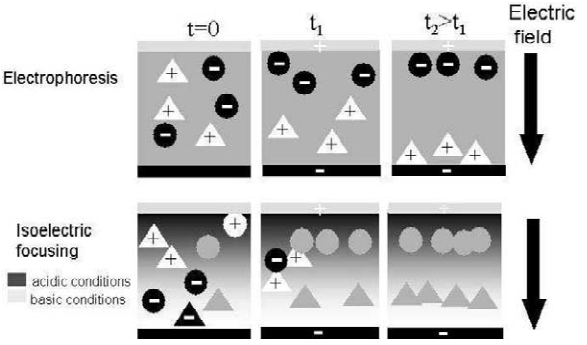


Fig. 9. Electrophoresis and isoelectric focusing.

fractionated on the basis of electrophoretic mobility, which is the ratio of the velocity of a particle in an electric field to the strength of that field. Alternately, electrophoresis can be combined with a sieving matrix, most commonly a gel, to fractionate particles on the basis of size if the particles have been treated to insure a uniform electrophoretic mobility.

One of the most common methods of analyzing proteins and DNA is capillary electrophoresis (CE), in which a homogenous solution is loaded into a high aspect-ratio channel. A field is applied length-wise across the channel, inducing charged particles to migrate towards the electrode of opposite sign. Once the fractionation has been completed, the channel contents can either be analyzed in situ or pumped to a downstream detector. Some of the possible advantages of a microfluidic implementation of CE over traditional CE have been summarized by Manz et al. and include higher efficiency, faster response time, and reduced reagent consumption [94]. These advantages are particularly important when working with clinical samples, which are typically complex and limited in both total volume and concentration [95]. Microfluidic devices offer the possibility of integrating sample preconditioning and analysis of small volumes.

Microfluidic CE systems using either pressure-driven flow or EOF have been successfully demonstrated. One of the first groups to demonstrate on-chip microfluidic capillary electrophoresis used a glass device with EOF-driven flow [94,20]. Electrophoresis on a planar glass microchip with laser-induced fluorescence detection was used to produce chromatographic separations of biological samples [96] with higher efficiency than in conventional fused-silica capillaries and comparable to gel electrophoresis methods. A microfluidic DNA-typing electrophoresis device was developed for an allelic profiling assay of short tandem repeats that is 10 to 100 times faster than capillary electrophoresis [97]. Electrophoretic separation of neurotransmitters and DNA restriction fragment and polymerase chain reaction product-sizing were demonstrated in a microfabricated glass-based system with integrated electrochemical detection [98].

Microfluidic CE has been used to analyze many classes of molecules beyond DNA. The affinity coefficient of a monoclonal antibody was measured using a microfluidic device to perform affinity CE [99]. Detection of phenols by a glass micromachined capillary electrophoresis system using indirect fluorescence was compared with a conventional capillary electrophoresis system using direct ultraviolet detection [100]. Two phenols were separated in two orders-of-magnitude less time, but with only one-third the precision. A protein biological-threat agent detection system that combines laser-induced fluorescence with a microfluidic electrophoresis device was developed and tested with the simulant, ovalbumin [101]. The achieved concentration detection limit was comparable to detection of 900 out of 4560 injected molecules. A microfluidic device in PDMS that employs a microavalanche photodiode was used to detect the separation of a mixture of proteins and small molecules by capillary electrophoresis [102]. The excitation light was brought to the microchannel by optical fiber and the photodetector, operated in Geiger mode, was close enough to the microchannel to make transfer optics unnecessary. A microfluidic system using capillary electrophoresis laser-induced fluorescence was developed for assessment of cell viability [103]. It was demonstrated with two bacteria and yeast in single and mixed samples.

Advances in 1997 in DNA and protein separations, cell manipulations, immunoassays and polymerase chain reaction using electrophoretic separation on microchips as part of a miniaturized total analysis system were reviewed by Kopp et al. [104]. Sample handling and separation in microchip-based electrophoresis was reviewed with emphasis on applications in human serum protein analysis, immunoassay, and DNA studies [95]. In 1999, Regnier

[105] examined the role of microchip-based separation systems in the evolution of chemical analyses in biology, medical research, and health care. The emphasis was on protein purification, peptide fractionation and sequencing, amino acid analysis, and DNA sequencing. Microfabricated electrophoretic devices were able to perform rapid genotyping assays and sequenced single-strand DNA in less than 15 min [106]. In 2000, Baldwin [107] reviewed three years of advances in capillary electrophoresis detection systems with a focus on the non-electrochemist audience. He includes devices based both on amperometry and on potentiometry and discusses the evolution toward microfabricated lab-on-a-chip analysis. In 2001, Krishnan [108] reviewed miniaturized reaction and separation systems including the microscale separation columns used to analyze DNA, RNA, proteins, and cells.

There are been many technological advances since the first microfluidic CE device. Thermoresponsive polymer matrices that change reversibly from high-viscosity solutions at 25 °C to low-viscosity colloidal dispersions at elevated temperatures were demonstrated in electrophoresis microchannels [109]. Matrix loading flows were accelerated by three orders-of-magnitude while still retaining the optimal sequencing performance of highly entangled solutions of high molar mass polymers. A microfabricated glass chip that integrates separation, staining, virtual destaining, and detection to perform a protein-sizing assay was demonstrated [110]. It used a novel electrophoretic dilution step to bring the sample complexes below the critical micelle concentration to reduce background and increase peak amplitude by one order-of-magnitude. Synchronized cyclic capillary electrophoresis was demonstrated in which a sample is separated during many cycles in a closed loop separation channel using a polygon geometry of 3 to 5 sides [111]. Optimizations included reduction of losses and dispersion at the intersections by designing voltage connections that are more shallow than the separation channel, and reduction of dispersion in the turns by narrowing the channels at the corners. A microfabricated separation system using photodefinable polyacrylamide gels as a sieving medium for DNA electrophoresis instead of non-cross-linked polymer solutions was shown to offer shorter curing times, locally-controlled gel interface, and simpler handling [112]. An electrode-defined sample compaction and injection technique achieved sample compaction without migration into the gel.

To develop a better way to introduce samples from the macro environment, a pressure-driven sample-introduction channel for a manifold of electrokinetic flow channels was designed and tested [113]. Leakage flow was less than 1% of the electroosmotic flow rate and the quantitative results obtained from electrophoretic separations in the electrokinetic channels were unaffected by the pressure-driven channel.

4.4.2. Isoelectric focusing

Isoelectric focusing (IEF) is similar to electrophoresis in that both techniques rely on the movement of a charged particle in an electric field. However, IEF selects on the basis of isoelectric point (p*I*), which is defined as the pH at which a particle has no net charge. To perform IEF, a pH gradient must be constructed between the two electrodes generating the electric field, oriented such that the acidic region is proximal to the anode and the basic region is proximal to the cathode.

Isoelectric focusing (IEF) of sample biological analytes, bovine hemoglobin and bovine serum albumin, was performed in microchannels to illustrate the potential of ''microfluidic transverse IEF'' for use in continuous concentration and separation systems [59]. An optically-based pH-gradient detection method and a one-dimensional model were developed to interpret the physics of the system [114]. The pH gradients were formed by electrochemical decomposition of water at 2.5 V and optically quantified by acid–base indicators. Zone electrophoresis and IEF were used in a continuous-flow microfluidic device to concentrate bacterial solutions [60] and protein (bovine serum albumin in a single ampholyte buffer) [115].

4.4.3. Dielectrophoresis

Unlike electrophoresis and IEF, dielectrophoresis (DEP) works on both charged and uncharged particles. In DEP, a non-uniform electric field is applied to the solution under investigation. This field results in a polarization of both the particles suspended in solution and the solution itself. If the particle is more polarizable than the surrounding medium, the induced dipole aligns with the applied field and the

particle moves towards regions of higher field strength; this effect is described as ''positive DEP''. If the particle is less polarizable than the surrounding medium, the induced dipole aligns against the applied field and the particle moves to regions of lower field strength (''negative DEP'') [116]. Since the direction of movement is relative to the strength, not the direction, of the field, DEP can be performed with either DC or AC fields.

Dielectrophoretic forces generated by microelectrodes on the bottom surface of microchannel were able to differentially levitate different types of cells in a dielectrophoretic field-flow-fractionation device [117]. Stem cells were displaced higher than breast cancer cells producing a separation efficiency of 99%. Human breast cancer cells were separated from normal blood cells by dielectrophoretic/gravitational field-flow fractionation by exploiting the difference in dielectric and density properties between the cell populations [118].

Linear traveling wave dielectrophoresis was demonstrated in a microchip with latex beads and rabbit heart cells [119]. The dependence of latex bead motion on applied potential and frequency, suspending medium conductivity, bead size, and surface characteristics was studied. Their surface conductance was determined from the dielectrophoretic crossover frequency.

An analytic solution was derived to study the effects of electrode spacing and electrode width on the gradient in electric field intensity produced by a two-dimensional array of parallel electrodes [120]. The intensity gradient was found to be dependent on electrode width squared and increase exponentially when the electrode spacing is less than two widths.

4.5. Proteomics

Biologically-based research has moved increasingly towards a ''holistic'' approach, leading to the development of genomics and proteomics [121]. Although genomics can provide significant information, protein expression is necessary to understand the function and regulation of an organism. The current standard for proteomics is the 2D gel, in which a complex solution of proteins is first fractionated on the basis of isoelectric point, via IEF, and then fractionated on the basis of molecular weight, typically using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) techniques [122]. In 2001, Figeys [123] surveyed the recent developments in microfluidic and array technologies that were being applied to proteomics. Lee [124] surveyed the tools available for proteome analysis including 2D protein electrophoresis, mass spectrometry, spotted-array-based methods, and microfluidic devices. He pointed out that additional technology was required to be able to fully integrate proteomic information with information obtained about DNA sequence, mRNA profiles and metabolite concentrations into effective models of biological systems. Jain [125] reported on advances in microfluidic devices for genomics, drug discovery and molecular diagnostics with a focus on biochip and microarray technology. In 2002, Mouradian [126] reported on advances in applications of microfluidic chips in proteomics including protein sizing, two-dimensional separation, and integration of trypsin digestion, separation, and injection into a mass spectrometer within a single device.

4.6. Sample preconditioning

A key component of successful assays is proper sample preparation and handling upstream of the assay itself. Very often the target analyte is initially present in low concentration and in solution with numerous other constituents that can impair assay function. Sample preconditioning, by removing these interferent constituents and concentrating the analytes of interest, can significantly improve the operation of the downstream assay. The macroscopic techniques for sample preparation such as precipitation, specialized membranes, and centrifuges are not directly amenable to microfluidics.

Other techniques, such as those based on electrokinesis, offer viable microfluidic solutions to the question of sample preconditioning. The successful concentration of proteins and vegetative bacteria has been demonstrated using free-flow continuous IEF in a laminate device driven with pressure driven flow [59,60]. A microchip-based sample purification technique was demonstrated by removing primers from a sample of amplified DNA [127], leaving only product DNA. A microchamber for electrophoretic accumulation of charged biomolecules was designed

for use as a preconditioning step in the analysis of DNA and protein samples [128]. Concentration of DNA oligomers and streptavidin in aqueous solutions was increased up to 200 times at the center of the chamber.

Simple differences in the diffusion speed of molecules with different molecular weights have also been utilized for sample preconditioning. Jandik et al. [15] describe microfluidics-based sample preparation prior to high-pressure liquid chromatography (HPLC). The H-Filter™ makes possible a diffusional transfer of an analyte from a sample stream into a stream of a ''receiver'' fluid (see Fig. 10). The authors discuss the extraction of an antibiotic cephradine from blood to demonstrate the utility of the new device. The new method completely avoids the use of centrifuges that is otherwise typical for most current methodologies for the preparation of blood samples prior to HPLC analysis.

Fig. 11 illustrates the position of liquids at the end of a sample preparation. The dark segment represents an unprocessed aliquot of the whole blood. As represented in the enlarged portion of Fig. 11, a diffusive mass transfer of cephradine and other low molecular weight compound is taking place (see Fig. 12). The red blood cells and other blood particles remain in the sample stream and are removed into the waste loop WL. Under optimized conditions, a large portion of high molecular weight compounds (i.e. proteins) also remains in the original sample and ends up in waste.

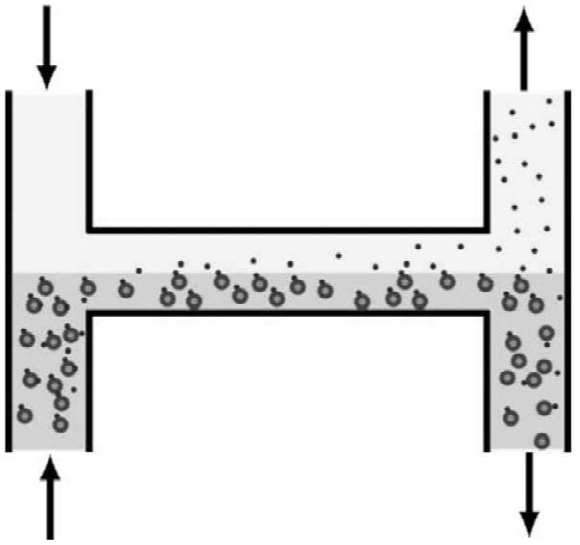


Fig. 10. H-Filter Schematic. Two streams are introduced into a common channel, where the form a laminar fluid diffusion interface (LFDI). Depending on their diffusion coefficient, particles start to diffuse across the LFDI, with the smaller particles diffusing more rapidly. Under optimized conditions, the majority of a population of small particles can be removed from a sample also containing larger particles.

4.7. Immunoassays

Using the LFDI technology discussed earlier, a rapid microfluidic diffusion immunoassay has been developed (see Fig. 13) and shown to detect subnanomolar concentrations of phenytoin, a typical small drug molecule used to treat epilepsy, in less than 1 min [6]. The assay was performed on diluted whole blood; no removal of blood cells was necessary. Results compared well to those obtained with a commercially available macroscopic assay. A competitive binding reaction is established in which a known concentration of labeled analyte is added to the unknown sample, which is then injected into the device and allowed to flow adjacent to a stream containing a known concentration of antibody to the analyte, permitted interdiffusion between the two streams. Once bound to antibody, the rate of diffusion of the analyte drops sharply, resulting in an accumulation of antibody–antigen complex at the interface between the two streams. The degree of accumulation, optically tracked with the labeled analyte, is modulated by the concentration of unknown analyte, which competes for binding sites with the labeled analyte.

A glass-microchip system for competitive immunoassay of serum theophylline was developed [129] that includes a mixing of sample with tracer step and a mixing/reaction with antibody step, both controlled by electroosmotic pumping, followed by an electrophoretic separation step. A microfluidic chip based on Borofloat glass performed electrophoretic separations of many samples over several months to measure affinity constants of monoclonal antibodies to bovine serum albumin [99].

Biotinylated lipopolysaccharide (LPS) was immobilized on streptavidin-coated sensor chips to study the binding affinities of different LPS-binding proteins and peptides [130].

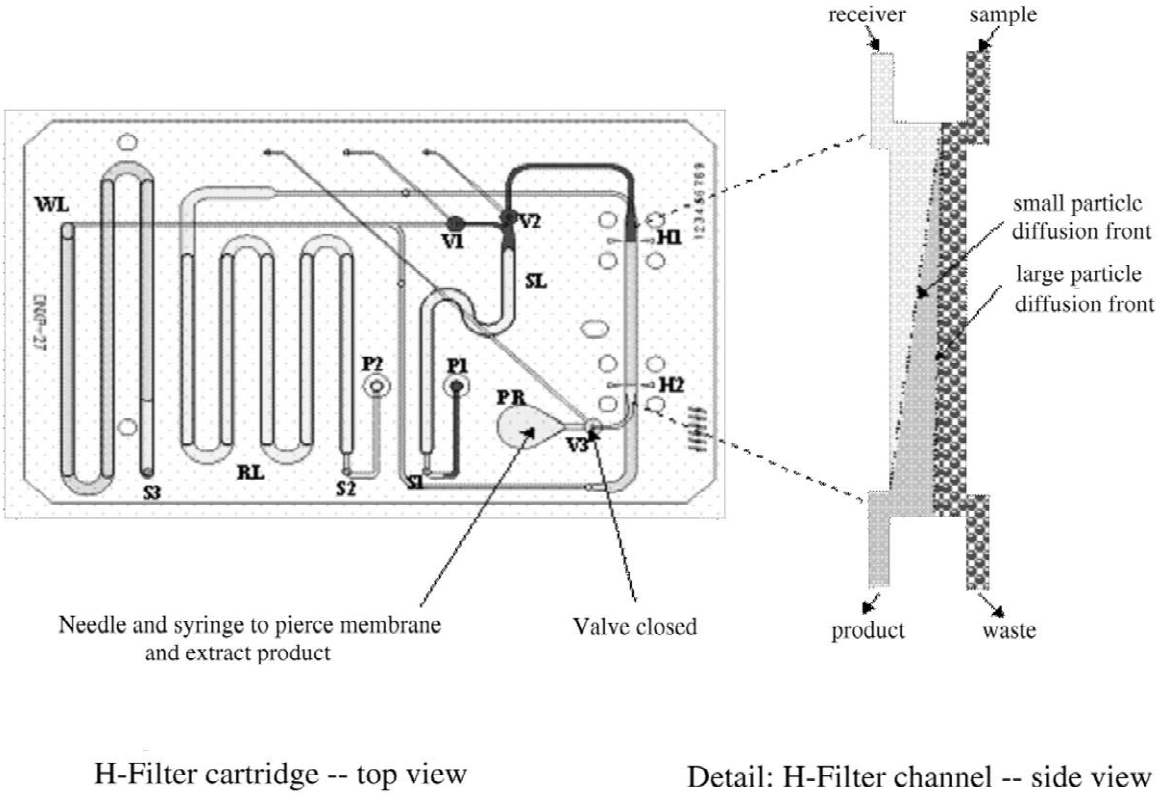


Fig. 11. Fluid positions at the end of a sample preparation in an H-Filter card.

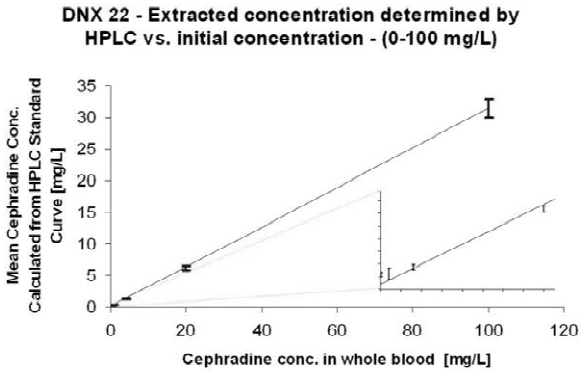


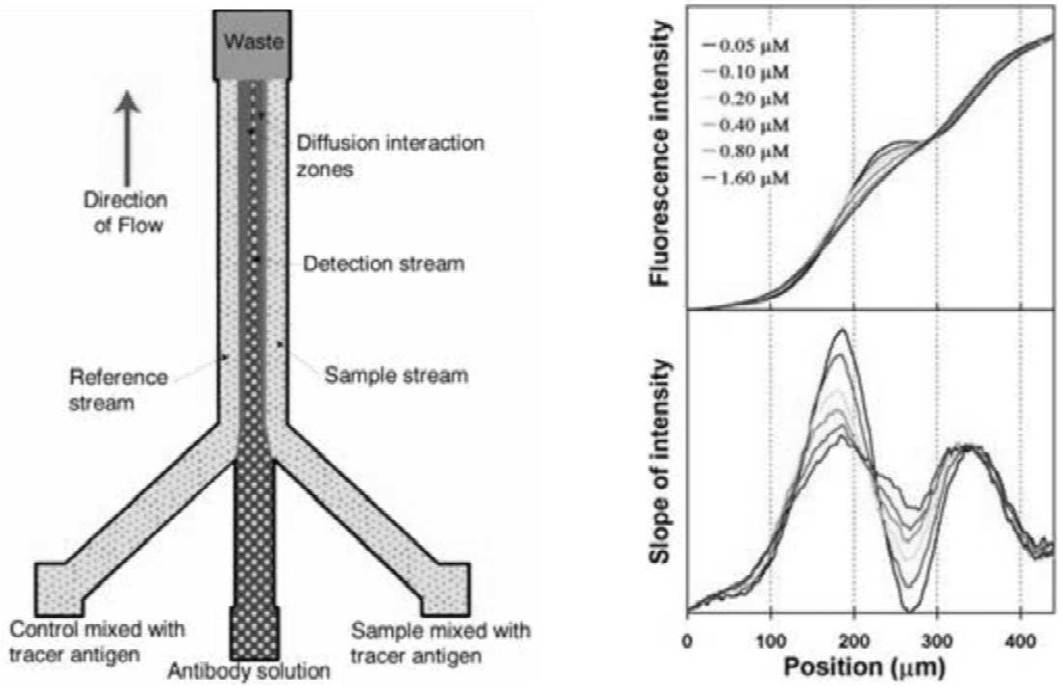
Fig. 12. Mean cephradine concentration as extracted by the H-Filter from whole blood without preconditioning.

4.8. Integrated multi-step systems

Ideally, all aspects of a medical assay, from sample injection to sample preconditioning and analysis, would be performed on the same device. Several groups have recently been moving towards such integrated systems. Voltammetric analysis with on-chip chemical reactions and fluid manipulations were demonstrated on a microfluidic device [131]. Manipulation of the electroosmotic flow enables stopped-flow and reversed-flow operations allowing extended residence times in the detector compartment.

A complete microfluidic system of DNA sizing and quantitation was developed, including: disposable glass microchannels, reagents, and software and instrumentation for electrophoretic separations and fluorescence detection of double-stranded DNA [132]. Analysis of polymerase chain reaction products, sizing of plasmid digests, and detection of point mutations were demonstrated.

A 330 picoliter chromatographic bed was fabricated in a glass microchannel by trapping 1.5–4.0 micron octadecylsilane-coated silica beads between



(a) Schematic (b) Calibration curve

Fig. 13. Diffusion Immuno-Assay (DIA) schematic and experimental results from a DIA for Phenitoin [6].

two weirs [133]. EOF was used to repeatedly exchange the beads through a bead-introduction channel and to sequentially introduce a non-polar analyte solution followed by acetonitrile to achieve a concentration increase of up to 500 times.

A microfluidic device that combines capillary electrophoresis with an integrated planar radio-frequency detector coil used for nuclear magnetic resonance (NMR) spectroscopy was developed [134]. Separations were demonstrated, but high concentrations were required to obtain satisfactory NMR spectra.

Electrokinetic pumping was used in a microfluidic chip with biospecific layer immobilized on the reaction chamber walls [135] (protein A with fluorescently labeled rabbit IgG as sample). Concentration gains of 30 of antibody from dilute solution onto solid phase were shown for concentrations down to 50 nM.

Multiplexed enzyme assays were demonstrated in a microchip capillary electrophoretic device [136]. Screening one compound against multiple enzyme targets achieved identical performance to individually-performed assays.

Coupling two different pre-column enzymatic reactions of the same substrate followed by electrophoretic separation of the reaction products generated two electrophoretic peaks for a single analyte, glucose [137] (and the simultaneous measurement of glucose and ethanol [138]). The peak current ratio is useful for confirming the peak identity, estimating the peak purity, addressing co-migrating interferences, and deviations from linearity. A similar microdevice that adds a post-column reaction demonstrated a detection limit of 2.5×10^{-16} g/ml for a mouse IgG model analyte [139].

An quartz electrophoretic microchip was demonstrated that can be directly coupled to pressure-driven flow analyzers with no electrical potential applied because the pressure-filled sampling channel of the microchip is kept floating [140]. Dye was injected into the sample stream in continuous or

discrete mode, then separated and detected on-chip by laser-induced fluorescence.

A microfluidic immunoassay device that integrates six independent mixing, reaction, and separation manifolds and two optical alignment channels for the scanned fluorescence-detection system was developed [141]. It offers direct immunoassays of six samples simultaneously or, even more interesting, simultaneous calibration and analysis.

A microfabricated glass electrochemiluminescence detector for micellar electrokinetic chromatographic separation was demonstrated using the legs of a ''U''-shaped floating platinum electrode placed across the separation channel as the working and counter electrodes [142]. The high voltage at the detector did not interfere with the electrochemistry.

4.9. Sample preparation for mass spectrometry

Mass spectroscopy is a very sensitive and versatile detection technique that is now being applied more and more in the biological sciences, especially in high-throughput genomics and proteomics. However, the technique requires that samples are provided in a reproducible and ionizable format. In electrospray and Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectroscopy applications, microfluidic devices have been used in sample preparation circuits to remove interacting particles and ions from the sample prior to ionization.

A new company, Gyros, AB, is producing commercially available integrated microfluidic systems for MALDI, including sample volume measurement and sample preparation (desalting, elution, and crystallization) [143]. These devices are fabricated onto compact disks, in which processing steps are located sequentially along a radius from the outer edge of the disk towards the center. Using this configuration, copies of a many-step process can be arrayed radially, allowing for parallel processing of multiple samples. In these devices, fluid flow is driven either by capillary action or centrifugal force. A second company, Gamera (now part of Tecan), also uses rotational motion of the entire device to mediate fluid transport and mixing. Their product, the LabCDTM, relies on a program of rotational velocity variation, in combination with chip design that includes high- and low-resistance channels, to create automated protocols with fixed incubation times and fluidic flow rates.

Microfluidic modules were assembled to combine electroosmotic pumping of solvent gradient, a reverse-phase microcolumn, and an electrospray ionization ion trap mass spectrometer for analysis of peptide mixtures from proteolytic digestion of proteins [144]. Filters in the reservoirs and exit of the microfluidic system and a mount to connect it to the electrospray mass spectrometer were developed [145]. Chemical noise observed by the mass spectrometer was greatly reduced. A polymeric microfluidic chip made of Zeonor 1020 provided direct electrospray mass spectrometric detection of polar small molecules separated by capillary electrophoresis [146]. No surface treatment of the polymer was required. Emitters for electrospray have also been microfabricated in polydimethylsiloxane [147–149]. Another integrated monolithic device combined an electrospray interface to a mass spectrometer with a capillary electrophoresis channel, an injector and a protein digestion bed with immobilized trypsin on 40–60 micron beads [150]. Digestion, separation and identification of melittin, cytochrome *c* and bovine serum albumin were demonstrated. Coupled capillary electrophoresis and mass spectrometric techniques have been used to measure the values of the pK_a of the amino groups of the aminoglycoside antibiotic amikacin and of its acetylated derivatives [151].

Two microfluidic devices were reported by Jiang [152]: one to detect aflatoxin via electrospray ionization mass spectrometry, and a second to detect phenobarbital antibody and barbiturates. The microscale of these devices increased detection sensitivity significantly by analyte preconcentration during the affinity purification step, limited analyte dilution in the microdialysis junction, minimized sample loss, and allowed the use of nanoscale sample flow rates.

A method and microfluidic device to isolate a single cell by integrating a laser trap and dielectrophoresis was reported [153]. The target microbe was trapped at the focal point of the laser and excess microbes were moved away electrokinetically before the target was transported to and taken out from the extraction port.

A microfluidic module for nanoelectrospray ionization-tandem mass spectrometry was fabricated

in poly(methylmethacrylate) and tested [154]. Protein identification from small samples was comparable to commercially available nanospray capillary tips.

A microfluidic device for protein identification was developed that combines a membrane reactor for protein digestion with electrospray ionization mass spectrometry and an intermediate step of transient capillary isotachophoresis/capillary zone electrophoresis for further concentration of the peptide mixture, if needed [155]. A nanogram of protein sample is sufficient and can be analyzed in minutes.

4.10. Polymerase chain reaction

The polymerase chain reaction (PCR) is a sensitive method for DNA analysis [156,157]. A target DNA sample is incubated with primers targeted towards a specific DNA sequence or set of sequences and with all the materials needed to make new DNA strands. This mixture is cycled through a series of changes of a given experimental parameter, typically temperature, that controls the degree to which the DNA is double- or singly-stranded. After each cycle the number of copies of the target DNA sequence is doubled, thus allowing significant amplification of the initial DNA sequence concentration.

PCR was one of the first diagnostic techniques to be successfully implemented in a microfluidic format. The rate at which PCR can be performed is typically limited by instrumentation [158]. Microfluidic implementation of PCR decreases cycle time due to improved control of solution temperature. The high surface-area-to-volume ratio of microfluidic devices facilitates rapid heat transfer and smaller sample volumes have less heat capacity, allowing rapid temperature change.

One of the first groups to perform a successful microfluidic PCR combined the output with CE so that the output of the PCR reaction could be analyzed [159]. More recently, a micromachined chemical amplifier, operated with continuous flow, demonstrated a 20-cycle polymerase chain reaction (PCR) amplification of a 176-base pair fragment [158]. In this device the sample moves through regions of various temperature, rather than changing the temperature of a stationary sample. PCR amplification of genomic DNA targets from white blood cells captured behind weirs in the flow chamber was demonstrated from whole blood in silicon-glass microchips [160].

A moderate density array method using DNA chips arrays was developed for diagnostic sequencing of PCR products to reduce their cost and complexity in use [161]. Antibiotic resistant clinical isolates were visually detected within 1 h after PCR amplification. Another application is the DNA sequence-based identification of toxic medicinal plants used in Traditional Chinese medicine [162]. In 2001, Petrik [163] reviews the use of microarray devices originally developed for genomic projects for mass screening of blood donations for hepatitis C virus.

An integrated system for PCR analysis was attained by combining dual Peltier thermoelectric elements with electrophoretic sizing and detection on a microchip [164]. Using a DNA concentration injection scheme enabled detection of PCR products within as few as ten thermal cycles.

A microfluidic cartridge was developed to prepare spores for PCR analysis by sonication, addition of PCR reagent to the disrupted spores, and insertion of the mixture in a PCR tube [165]. The processing and detection of the spore DNA was completed within 20 min.

An integrated microfluidic device was demonstrated that combines stochastic PCR amplification of a single DNA template molecule followed by capillary electrophoretic analysis of the products [166]. A histogram of the normalized peak areas from repetitive PCR analyses revealed quantization due to single viable template molecule copies in the reactor.

A microfluidic chip for detecting RNA amplified by nucleic-acid-sequence-based amplification (NASBA) was developed [167]. Samples of cryptosporidium parvum were detected and clearly distinguishable from controls without separating the amplified RNA from the NASBA mixture.

An automated microfluidic system for nanoliter DNA analysis directly from cheek cells was demonstrated, including all the steps needed for DNA analysis: injection, mixing, lysis, PCR, separation, sizing, and detection [168]. The possibility of further miniaturization of the system was established.

An integrated microfluidic chip-based system was developed for quality control testing of a recombinant, adenoviral, gene therapy product [169]. The viral identity test sized and quantified the DNA

fragments and required 100-fold less sample than the agarose gel method.

Polyimide microchips with a 1.7 ml chamber containing a thermocouple were used to amplify a 500-base-pair fragment of lambda-phage DNA demonstrating infrared-mediated temperature control [170]. Adequate amounts of PCR product were obtained in 15 cycles, an amplification time of 240 s.

4.11. Novel concepts and applications

A fluidic analogue of the field-effect transistor was demonstrated with electro-osmotic flow inside a microfluidic channel controlled by an electric field perpendicular to the fluid flow [171]. Two of these analogues combined with a channel junction were able to generate opposite flows inside a single flow channel driven by electro-osmosis demonstrating the ability to control and switch flows in a microfluidic network.

A microfabricated capillary electrophoretic device that performs chiral separations of fluorescein isothiocyanate-labeled amino acids was developed for the extraterrestrial search for life [172]. The device obtained enantiomeric ratios of amino acids from the Murchison meteorite that agree with those obtained by HPLC.

Aqueous liquid droplets were encapsulated in a hydrophobic powder creating ''liquid marbles'' that were easily moved by gravitational, electrical, and magnetic fields [173]. Quick displacement of the droplets without leakage was demonstrated.

A fluid-temperature measurement technique using a temperature-dependent fluorescent dye was demonstrated by measuring temperature distributions due to Joule heating in electrokinetically-pumped microfluidic circuits [174]. Temperatures from 25 to 95 °C were measured with thermal, spatial, and temporal resolutions of 0.03°, 1 micron, and 33 ms, respectively.

5. Advances in modeling and analysis

Because of the small physical dimensions of microfluidic flows, it is difficult to insert sensors that do not disrupt the flow. Thus experimental techniques often measure bulk, instead of local, properties. Mathematical modeling, once correlated to experimental results, is useful for quantitative assessment of local behavior as well as for device design optimization. The fluid dynamics of nanoscale flow and mass transport have been studied by Ciofalo [175]. Computational models, especially for particle-fluid problems, are available for a variety of length scales.

Many analyses focus on various aspects of electrokinetic flows. Mammen [176] points out that plots of absorbance versus 1/time could replace the traditional plots of absorbance versus time, since electrophoretic mobility is linearly proportional to 1/time and thus the spacing between peaks corresponds linearly to differences in mobility. Locally-asymmetric electric geometries were proposed by Ajdar [177] for pumping liquid in microchannels and driving drops on surfaces. Fluid velocities were estimated by a simple electroosmotic model.

Electrokinetic injections on microfluidic devices were studied by computer simulations to identify operating parameters that provide optimal performance of the virtual valving [178]. In addition to study of pinched injection, gated injection was simulated and compared to experimental data to achieve leak-free valving during sample load. A variety of sample loading profiles and dispensing schemes for pinched injection were studied experimentally [179]. The axial extent of the sample plug decreased as the electric field strengths in the sample and sample waste channels were raised relative to the analysis channel.

Electroosmotic flow in microchannels with patterned surface charge was investigated experimentally and theoretically assuming thin double-layers and low surface potential [180]. Surface charge variation perpendicular to the driving electric field generated multidirectional flow along the direction of the field; charge variation parallel to the field generated recirculating cellular flow.

The effects of fluid inertia and pressure on the velocity and vorticity field of electroosmotic flows have been analyzed [181], assuming an inner flow region dominated by viscous and electrostatic forces and an outer flow region dominated by inertial and pressure forces. If conditions of uniform surface charge, low Reynolds number, low Reynolds and Strouhal number product, uniform fluid properties,

and zero pressure differences between inlets and outlets are true, there is full-field similarity between electric and velocity fields in arbitrarily shaped, insulating wall microchannels.

A number of interdependent factors that affect bulk flow in a microchip environment and contribute to peak shape, migration time, and baseline drift anomalies observed in electroosmotically driven separations were investigated [182]. Pressure-driven backflow was measured as an order-of magnitude larger than predicted from the pressure-head due to difference in liquid levels. Pressure from surface tension effects was implicated.

Ajdari [183] reports on the importance of lubrication approximation analysis to study of transverse electrokinetics and microfluidics in micropatterned channels. Using linear-response theory, applied pressure gradient and electric field can be related to flow and electric current for design of pumps, mixers, or flow detectors.

6. Future directions

The authors believe that microfluidic devices are beginning to make significant contributions to biomedicine and drug discovery. Conference attendance to microfluidics conferences and the rate of publications on the topic per year have increased exponentially. More than 2000 papers on the subject had been published by early 2000. The first generation of microfluidics-based analytical devices were already functional in 1999, with many more in active development. However, some of the promises of microfluidics (integration of all laboratory functions on a chip, the commercialization of truly hand-held, easy-to-use microfluidic instruments) have yet to be fulfilled.

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