Table 4 Statistical optimization schemes used in MEKC

Optimized parameter	*Parameters varied*	*Modelling*
Selectivity and resolution	pH, [SDS], [borate]	CCD[c], desirability functions
Selectivity and resolution	pH, [SDS], [sodium cholate], [AMPSO][a]	CCD, desirability functions
Resolution	[acetonitrile], [urea]	Iterative regression strategy
Resolution	9 for a stepwise screening, followed by 3: pH, [SDS], [acetonitrile]	Fractional factorial design, full factorial design, RSM[d]
Yield for the derivatization of some dipeptides	Reaction time, T, ionic strength, pH, [isopropanol]	Fractional factorial design, CCD, RSM
Selectivity	pH, [SDS]	Iterative regression strategy
Resolution	[SDS], [acetonitrile]	CABRO II[e]
Precision and efficiency	[SDS], V, T	FUMI[f]
Resolution	T, V, ionic strength, [SDS], [HPMC][b], [β-cyclodextrin]	PLS[g]
Resolution	[SDS], [urea]	CABRO II
Resolution	pH, [SDS]	CAMOS[h]
Resolution	pH, [buffer], [SDS], [SDS + sodium heptyl sulfate], [acetonitrile]	Plackett–Burman statistical design
Resolution	[SDS], [*N,N*-dimethylformamide], ionic strength	ORM[i]
Resolution	pH, [SDS], [tetrabutylammonium salt]	ORM
Resolution	pH, [SDS]	ORM
Resolution	[SDS], [isopropanol], [β-cyclodextrin]	Full factorial design
Resolution	pH, [SDS]	Full factorial design

[a]AMPSO = 3-[(1,2-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonicacid; [b]HPMC = hydroxypropyl methylcellulose; [c]central composite design; [d]response surface modelling; [e]computer-assisted bivariate resolution optimization II; [f]function of mutual information; [g]partial least squares; [h]computer-assisted multivate optimization strategies; [i]overlapping resolution mapping.
V, voltage; T, temperature.

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Microtechnology

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Introduction

Electrophoresis is an established separation technique, frequently used for mixtures ranging from proteins and DNA to small anions and cations. However, perhaps its greatest strength lies in its remarkable ability to separate charged macromolecules. Reports describing electrophoretic separations started to appear in the 1930s, but the most significant developments really took place in the 1940s and 50s when separations with a paper or gel support matrix were used for the separation of macromolecules. The early

methods used relatively large scale apparatus, but during the later 1960s, and early 1970s, reports appeared describing separations being performed in small bore tubes filled with buffer solution. This work was extended in the early 1980s, with capillaries being a key feature of the basic methodology. This was the start of capillary electrophoresis (CE); however, it was not until the mid 1980s that great interest was shown towards a new approach to separation science. From that moment, development and commercialization came very quickly and soon there were a number of commercial instruments available for routine laboratory use.

It is not possible to cover all aspects of electrophoresis in an article such as this; indeed there are several topics that have been omitted. Fluid logic devices and freeze–melt switching are two such examples; another important area not included is the use of parallel bundles of microcapillaries that permit multiple analyses to be performed at a high throughput.

The basic element of any CE system is the separation capillary, typically 10–100 μm internal diameter and 30–100 cm long. Each end of the capillary is located in a small reservoir, which contains buffer solution and a platinum anode or cathode; typically potentials of up to 30 kV can be applied between them. Detection is achieved by a range of in-line detection methods, such as ultraviolet absorbance and other detection methods, such as mass spectrometry, can be interfaced to the capillary.

Separation is achieved due to the differing electrophoretic mobilities of the analytes in the sample, but in addition electroosmotic flow (EOF) takes place. This phenomenon gives rise to bulk flow of the solution in the capillary without the need for an external pump. For a unmodified silica capillary, the direction of flow would be from the anode to the cathode, which enables all uncharged species to be carried to the detector. This technique offers very high separation efficiencies and rapid analysis. This feature, coupled with the simplicity of the instrumentation, makes the technique ideally suited to miniaturization.

Interest in miniaturizing analytical systems in not new; indeed, the idea of a micro total analysis system (often referred to as μTAS) has been mooted for some time within the scientific community (see, for example, the paper by Martin cited in Further Reading). The ideal approach is to include sample manipulation and detection on a chip-sized device; this has given rise to the term 'lab on a chip'. Such systems employ microstructures fabricated on glass or other substrates to form integrated devices rather than attempting to construct miniaturized systems from discrete components. However, there is also considerable interest in the development of discrete components, such as micropumps. The conference proceedings from the recent Micro Total Analysis Systems '98 give some indication as to the diversity of the developments. While on-chip injection is feasible, some prior degree of preparation may still be necessary. For example, particulate matter would quickly block the channels, so pre-filtering would be required in such situations. Before examining in more detail electrophoresis on chips, it is important to consider the fabrication of such microchannel devices.

Fabrication of Electrophoresis Devices

There are numerous fabrication methods available, and the complexity of possible designs is virtually limitless. A popular fabrication technique is the use of photolithographic masking in conjunction with wet or chemical etching. The simplest case would be the fabrication of a single channel in a piece of glass. First, the glass would be coated with a layer of deposited metal and subsequently photoresist, e.g. by spin coating, then the pattern mask is placed on top of the photoresist. This masked surface is subsequently exposed to ultraviolet light, which transfers the pattern on to the photoresist. The unprotected area can then be removed, along with the underlying metal surface. An etching solution, such as hydrofluoric acid/nitric acid, is used to etch away the glass, forming the channel in the chip. This surface of the chip protected by the metal and photoresist layer does not etch. This process can be seen in **Figure 1**. There are a few problems with this approach; the first is that only certain materials can be etched. The second is that, as the channel is etched deeper, the width also increases. This becomes more of a problem as the depth increases, resulting in channel with nonvertical sides. This problem can also create difficulties at channel intersections, which do not have true intersecting corners due to the accelerated etching of the exposed corners.

There are many alternatives to the wet etch approach. Dry etch processes include reactive ion and laser etching; these offer a way to cut precise channels of small dimensions. Silicon is gaining in popularity as an alternative substrate to glass for chip fabrication, and by employing more than one etching technique complex devices can be produced.

In order to use polymeric materials, such as silicone rubber, or fluoropolymers for chip fabrication, new approaches are required. This may take the form of stamping, imprinting or injection moulding of the polymeric material. The approach offers a significant alternative to wet etching of channels directly,

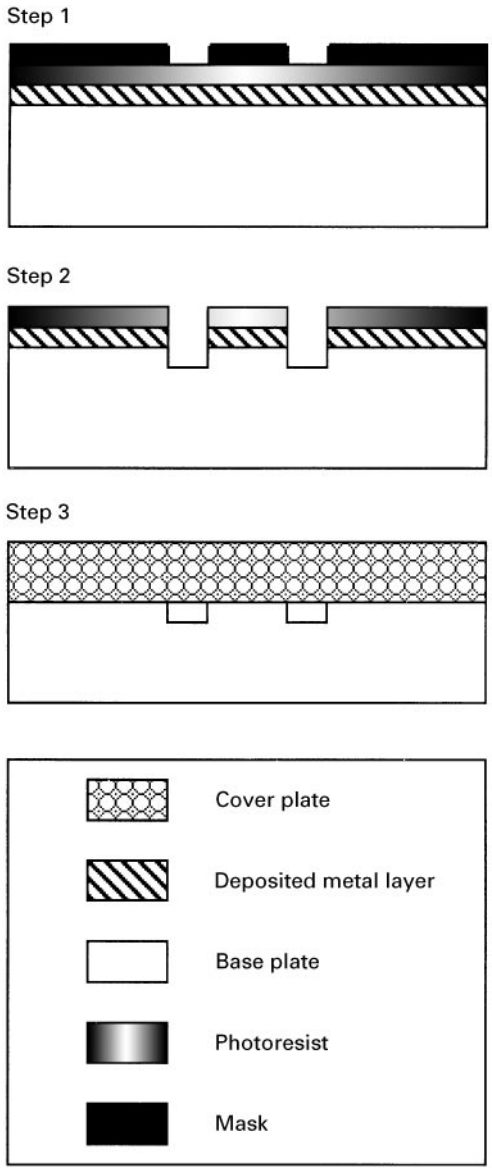

Figure 1 The fabrication process for a separation chip fabricated from silica. The first step is to place the mask on top of the silica base plate covered in deposited metal and photoresist (step 1). After this has been exposed to UV light, the chip is developed to remove the exposed photoresist and metal. It is then etched, e.g. with hydrofluoric/nitric acids (step 2); etching does not occur where the metal and photoresist remains. The final stage (step 3) is the bonding of the cover plate on to the base plate. The two etched channels can clearly be seen.

primarily since it allows the use of a wide range of new materials, and the prospect of mass production. It requires a template to be constructed, often by wet etching or mechanical milling. This template can be considered as the negative image of the channels, and is often finally produced in a more durable material, such as nickel. From this robust template, it is possible to mass-produce thousands of channel systems with considerable speed.

All of these methods create half the chip; the next step is to attach the cover plate, i.e. the other half of the chip. It is common to locate the holes for the necessary reservoirs in this plate; the reservoirs themselves are frequently constructed by attaching cylinders, e.g. truncated pipette tips, to the top plate. For glass and silica-based systems, it is a simple step to bond the top plate on to the channels by a heating and cooling cycle (the cooling cycle is required to avoid thermally stressing the glass). The fixing of the top plate to polymeric materials can be more complex; however, perhaps the simplest method is to use a thermally activated adhesive to laminate the top plate on to the chip. Typical channel dimensions are 200 μm wide by 60 μm deep, and vary in length from 5 mm to several centimetres. Of course, many other channel dimensions can be created. Some typical patterns can be seen in **Figure 2**.

Theoretical Considerations

There are two important effects that need to be considered when discussing electrophoresis in microchannels; these are similar to the more conventional capillary electrophoresis. The first is electrophoretic mobility, and the second is electroosmotic flow (EOF). EOF is otherwise referred to as electroendosmotic flow.

Electrophoretic Mobility

This process forms the basis for the separation in the channel, and dictates the migration velocity of a given ion in the channel. The electrophoretic mobility (μ_e) is related to the migration velocity (v) by eqn [1], where E is the electric field strength:

$$v = \mu_e E \qquad [1]$$

The units of μ_e, v and E are $cm^2\,V^{-1}\,s^{-1}$, $cm\,s^{-1}$, and $V\,cm^{-1}$ respectively. The electrophoretic mobility is proportional to the ionic charge and frictional forces. Thus, if two mobile species differ in either their charge or the frictional forces, then separation will occur. Since uncharged molecules have an electrophoretic mobility of zero, movement will not occur; this is why electrophoresis cannot separate neutral molecules. For ions of the same size, μ_e will be greater for ions with greater charge while for ions of the same charge, μ_e will be greater for smaller ions.

Electroosmotic Flow

This is a process which gives rise to the flow of buffer through the channel. It can be quite significant,

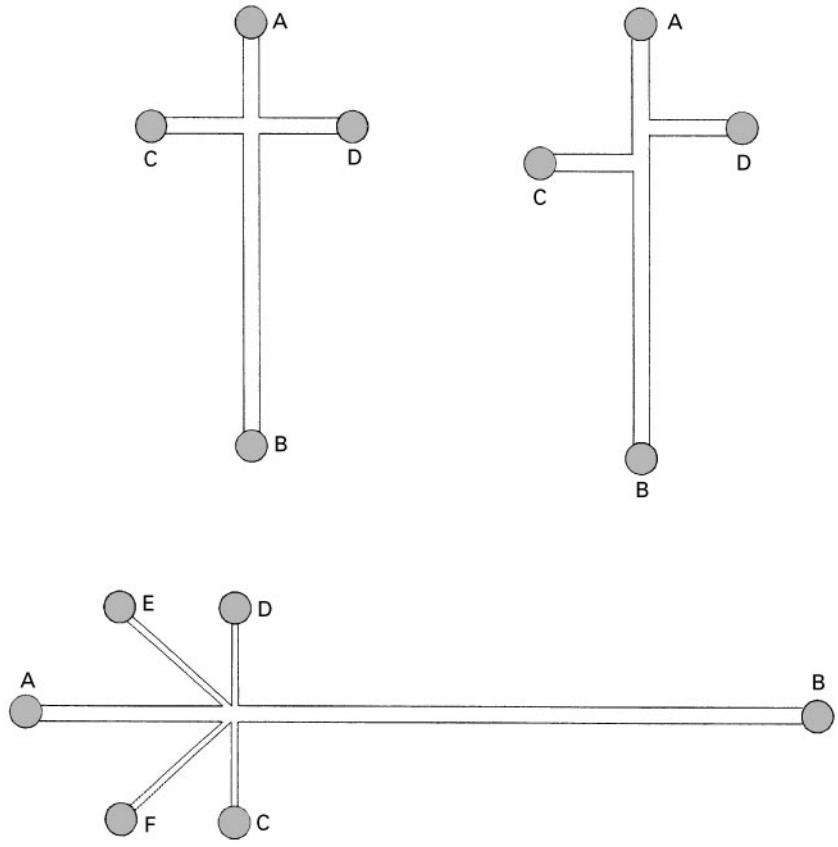

Figure 2 Some typical channel arrangements. Reservoirs A and B start and terminate the separation channel. Reservoirs C and D permit a known amount of sample to be injected into the separation channel. The reservoirs E and F permit the addition of other reagents to the separation channel.

reaching linear velocities of around 5 cm min^{-1} or greater. The rate of movement due to EOF is normally greater than the electrophoretic mobility, thus ensuring that all ionic (and uncharged) species pass the detector. However, unlike electrophoretic mobility, EOF will only occur in the presence of an electrical double layer at the surface of the channel. In **Figure 3**, an axial view of a channel etched in glass can be seen; the surface is covered in silanol groups.

When the pH of the buffer is above $\sim$ pH 9, all the silanol groups are ionized. Cations from the buffer migrate towards the negative wall of the channel, and a double layer is formed. When a voltage is applied across the channel, these cations migrate towards the cathode, thereby inducing bulk flow. Electro-driven flow has a characteristically flat profile compared to the parabolic profile observed for pressure-driven systems. This significantly reduces the dispersion due to flow, and is considered to be a reason for the high efficiency separations possible. Another reason for the low dispersion observed is that the Reynolds numbers for liquids in such a system are very low, which results in limited dispersion. The electroosmotic mobility (μ_{EOF}) is given in eqn [2] where η is the viscosity of the buffer, ε is the dielectric constant of the buffer and ξ is the zeta potential (charge on the capillary wall):

$$\mu_{EOF} = (\varepsilon\xi/\eta) \qquad [2]$$

The EOF velocity can be calculated from eqn [3] which has striking similarities to eqn [1]. Here, the EOF velocity (v) is related to the electroosmotic mobility (μ_{EOF}), and the electric field gradient (E):

$$v = \mu_{EOF}E \qquad [3]$$

From this, it is apparent that the overall velocity of the ionic species is the algebraic sum of the migration velocity, and the EOF velocity. By summing the two velocity terms and subsequent rearrangement of the equation, the actual velocity (v_a) of an ionic species is given by eqn [4]:

$$v_a = (\mu_E + \mu_{EOF})E \qquad [4]$$

Situations do arise, such as during the analysis of anions with high electrophoretic mobility, when the

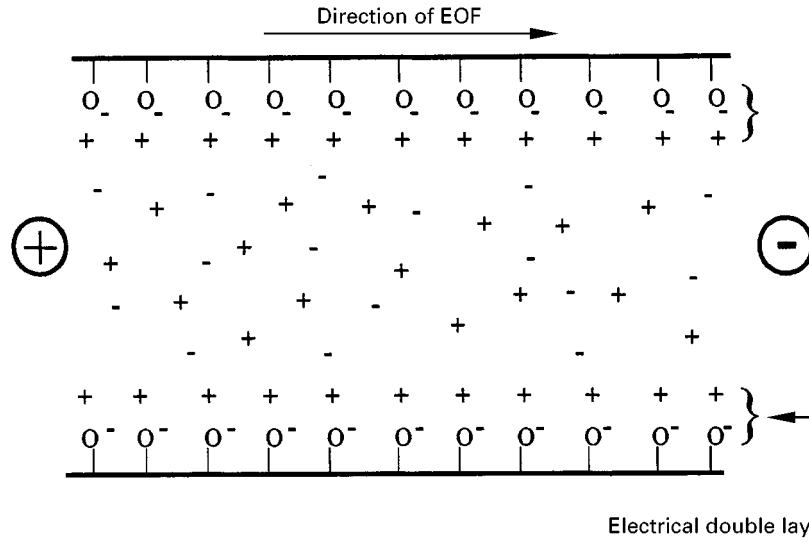

Figure 3 The double layer formed in silica channel. The layers of cations which collect along the walls of the channel will migrate towards the cathode when a voltage is applied. This gives rise to the electroosmotic flow (EOF) with the characteristic flat flow profile.

direction of EOF needs to be reversed. This can be achieved by coating the walls of the channels with a cationic surfactant. This gives an apparently positive charge to the walls, so that anions (not cations) will form the double layer. Then, when the potential is applied, EOF will be in the opposite direction. Since the influence of the double layer is generally considered to extend less than 1 μm into the solution, overlap of the double layer should not be an issue for channels of greater than 5 μm minimum dimension. However, for channels of smaller dimension, the flat flow profile model may no longer be valid, and great care should be exercised in describing the flow.

To prevent EOF completely, the walls of the channel need to be rendered neutral. In silica channels, this ought to be achievable by coating the walls with a compound such as trimethylchlorosilane, to end-cap all terminal silanol groups. However, in practice, it is impossible to eliminate all EOF since residual surface charge remains. Since many microsystems are now being constructed from polymeric substrates, EOF normally does not occur to any appreciable extent. This is due to the absence of ionizable or charged surface groups. In this situation, EOF could be induced by coating the walls of the channel with a charged compound, such as cetyltrimethylammonium bromide.

Practical Considerations

Perhaps the key practical consideration is whether integrated on-chip detection will be employed, or whether the separated compounds will be transferred to another device, such as a mass spectrometer. In a similar context to conventional capillary electrophoresis separations, on-chip detection is the ideal option, since it minimizes dispersion and the dead volume associated with the transfer of analytes from the chip to a detector. The dead volume will normally be far in excess of the separation volume, thus band broadening will be a serious problem.

The other key issue is sample introduction. The simplest system relies on the EOF to introduce the sample into the separation capillary. Consider the channel arrangement in **Figure 4**. The channels are etched into silica, and no deactivating treatment is applied. Under normal conditions (I), the applied voltage between reservoirs A and B induces EOF. In addition, the potential field gradient will give rise to electrophoretic separations.

Since only buffer is flowing, this does not give rise to any apparent separation effect. When the voltage is manipulated such that it is now between reservoirs C and D (II), EOF is induced between the reservoirs, thus the sample is introduced, and occupies a small section of the main channel. Once the voltage is restored between A and B, the separation step begins (III). Here, the sample is moved by the EOF towards reservior D, and separation occurs due to electrophoretic mobility.

In situations where EOF is insignificant due to the absence of surface charge, the injection step relies either on the electrophoretic movement of the analytes or an applied pressure. There is, of course, a potential problem with electrophoretic mobility, and that is the discriminatory effects observed between analytes of high and low electrophoretic mobility. Pressure, on the other hand, offers a simple

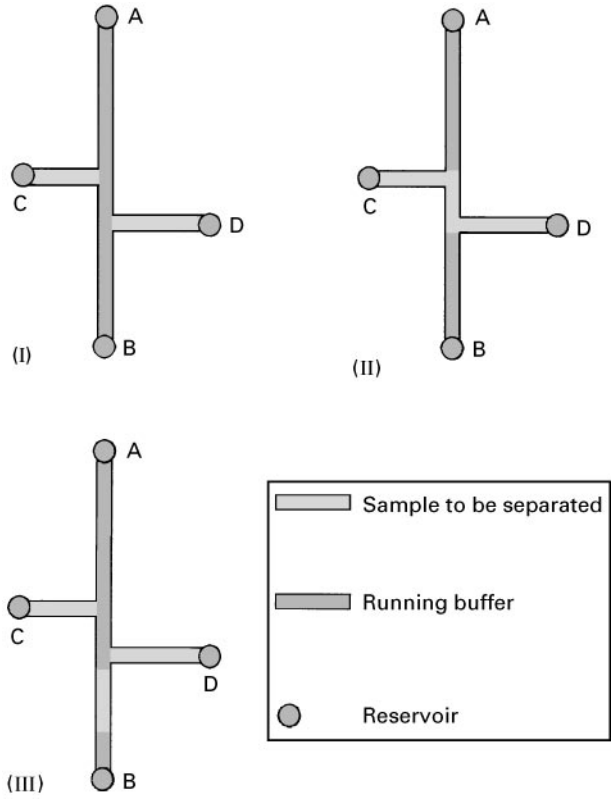

Figure 4 Sample introduction into the separation channel. (I) When the voltage is applied between reservoirs A and B, the separation channel is filled with running buffer. (II) To inject a sample, the voltage is applied between C and D: the sample moves into a short section of the separation channel. (III) With the voltage restored across A and B, the sample moves along the separation capillary, and separation occurs.

and nondiscriminatory route for sample introduction. This can be achieved by either applying pressure to one reservoir in order to force the analyte through the system, or by deformation of the chip (in situations where the polymer is flexible). In either case, a valveless injection method is used; this greatly simplifies the operational aspect of these systems.

Applications

In this section, several types of application will be considered. While much of the discussion will be related to the separation of compounds on chips using electrophoresis, it is impossible to neglect the potential of EOF alone for fluid mobility, which is unaffected by back-pressure.

Electrophoretic Separations

Much of the literature available on chip-based electrophoretic separations features capillary zone electrophoretic (CZE) separations; however, there are many other types of separation possible, such as isotachophoresis and electrokinetic focusing.

Perhaps the simplest applications are based on CZE within silica microchannels. Here EOF and electrophoretic mobility can be utilized, the EOF for injection and bulk flow of solutions through the capillary, and electrophoretic mobility for the actual separation process. A typical separation capillary would be 50 mm long, 45 μm wide and 8 μm in depth, with an applied potential in the range 600–1200 V along the 50 mm length. The types of samples that can be separated by this technique are extensive (not surprising, given the diversity of the applications for conventional CZE) but include small anions and cations, monoclonal antibodies, theophylline and DNA fragments. There are a number of potential detectors, but those based on optical or electrochemical methods are the most frequently used.

Electrochemical detection can easily be incorporated on to a microchip, but requires the detector to be located after the high voltage section of the channel. This is necessary to prevent the high voltage causing interference with the detection. This can be achieved in such a system as described above, by locating the electrochemical detector in the channel just after the ground electrode. The EOF occurring in the channel would pump the fluid along the channel from the ground electrode to the detector electrodes. Over this short region, band broadening should not pose a significant problem. It is similar in principle to the porous junction technique widely used in conventional CE. It is possible to achieve limits of detection of micromolar levels or better with electrochemical detection.

Spectroscopic methods fall into two main classes – absorbance and fluorescence. Absorbance measurements are simple to effect, but commonly suffer from relatively low sensitivity. This is primarily due to the channel dimensions resulting in a very small path length. Measurements across a 50 μm channel would give rise to a very small absorbance, since absorbance is proportional to path length. It is possible to increase the path length (**Figure 5**), but absorbance measurements do not have the sensitivity of fluorescence measurements, although they are generally applicable to a wider range of analytes. In addition, for practical reasons, dual-channel systems are not easily set up and this can lead to instability in the detector signal.

Fluorescence measurements can provide limits of detection in the picomolar range (varying from around 2 pmol L^{-1} upwards), and have even been reported for counting single chromophore molecules. Generally, the excitation source is directed along the

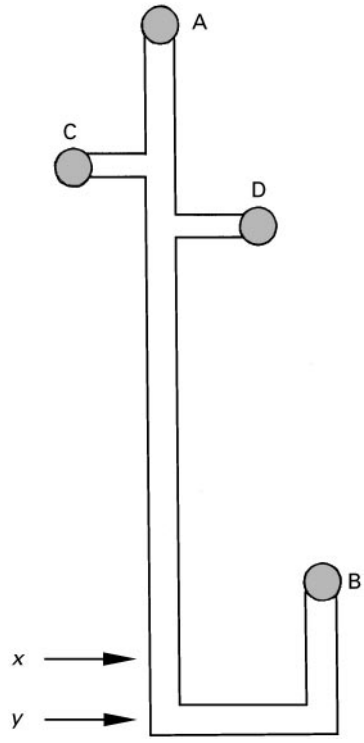

Figure 5 UV detection can be made more sensitive by increasing the path length of the measurement. When the absorbance of the analytes is measured across the channel at point *x*, the path length is equal to the channel width (typically 50 μm). By making the measurement at point *y*, the path length is equal to the channel length (typically 3–5 mm).

channel, in order to minimize the scatter from the walls of the channels. By careful alignment, it is possible to minimize the background, and obtain very sensitive measurements. There are many other detection methods, including optical waveguide sensors and chemiluminescence, but fluorescence detection currently offers the most sensitive analysis.

Similar results can be obtained with channels produced from polymeric support materials. There is one issue that must be addressed with certain materials, e.g. plastics; that is, the background fluorescence that is frequently observed. This can be due to the actual substrate, or the adhesive used to seal the chip. Careful selection of materials helps to reduce the problem. However, it is the prospect of the mass production of thousands of chips with hundreds of channels per chip from just one master template that is particularly attractive. Once mass production is achieved, the devices will become truly disposable.

Electrokinetic Induction of Flow

The factor often overlooked with microsystems is the value of EOF for fluidic manipulations. EOF will occur at potentials of 100–200 V cm^{-1}, and can be used to drive fluids through channels, and indeed physical objects such as cells, e.g. *Escherichia coli*. It is possible to transport whole cells around the channels on a microchip. In addition to the EOF, there will also be electrophoretic separations occurring, but in practice, these are small compared to the EOF on uncoated silica surfaces. To be of practical use, it is necessary to have the ability to make meaningful measurements on the contents of the cells. This can be most easily achieved by lysis of the cells with detergent. It would then be possible to measure compounds, which would otherwise have been trapped within the cell wall. Since the volume of the channels is small, the released compounds will not be extensively diluted, and the time to analysis will be very short; this is particularly important if the aim is to study rates of reaction or unstable compounds.

EOF serves to deliver the sample beyond the high voltage area if it is intended to use off-chip detection. For example, to transfer the separated compounds from a separation chip to a mass spectrometer, EOF can be used to deliver the compounds to an electrospray interface. Indeed, it is possible to generate the electrospray between the terminal end of the capillary and a suitably located conductor, without the need to apply a conductive coating to the end of the chip.

The Future

Electrically driven separations on microchips have a number of advantages over conventional CE. The first is the further reduction in reagent consumption, in terms of both sample and buffer solutions. This will reduce the running costs of the system, and also the associated waste disposal costs. Second, as methods to mass-produce the devices become more widespread, the cost will decrease. This will allow totally disposable systems to be used. Finally, perhaps the most important advantage will be the portability of analyser systems, which will be able to be used in remote on-site mode. Without doubt, microtechnology revolutionized the electronics industry, and it will do the same for much chemical analysis.

There remain two other areas where chips or micro reactors will find important uses, and that is in chemical discovery and manufacturing. Combinatorial chemistry can produce thousands of compounds per day, which places a significant demand on the analysis. The fabrication of multiple analysis channels with high spatial resolution may offer an analytical solution for this problem; however, appropriate detection must also be available. Mass spectrometry interfaced

to an electrophoretic separation chip is one possible answer. However, there is no reason why reactions cannot be carried out on such devices. Since it does not require the investment of a large chemical plant, the reactions can be performed where required, thus reducing the need to transport hazardous chemicals across countries. Since many reactors can be constructed on a single chip, and many chips located in the same area, it is evident that this technology will provide hazardous or chemically unstable chemicals where they are required.

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Nonaqueous Capillary Electrophoresis

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Electrophoresis is a separation technique that is normally performed in an aqueous environment. This is due to the fact that the separation mechanism is based on the difference in migration rate of charged species in an electric field. Species (ions/molecules or particles) with a difference in their charge over size ratio will exhibit a difference in migration rate. Most charged species are fairly soluble in aqueous media and thus water is the most obvious solvent for electrophoresis. However, in a number of nonaqueous solvent systems, it is possible to obtain sufficient conductivity to perform electrophoresis. If such systems are utilized with the technique of capillary electrophoresis, a number of advantages compared to aqueous systems are obtained in the separation of small molecules. Nonaqueous electrophoresis of biopolymers like polysaccharides, nucleic acids and proteins is not of practical use due to lack of solubility of such molecules in organic solvents.

Nonaqueous Capillary Electrophoresis

Only a few attempts to perform nonaqueous paper electrophoresis have been described and these articles were reviewed in 1978. In 1984 nonaqueous capillary electrophoresis (NACE) was briefly mentioned in a single publication, but not utilized further. However, since 1993 the use of nonaqueous media for capillary electrophoresis has seen renewed interest in the separation of drug substances due to the high separation selectivity obtained in these systems.

The electrophoretic migration of the solutes is influenced by the nature of the solvent or solvent mixture used for the electrophoresis medium in three main ways:

1. The mobility may change due to changes in the size of the solvated ion.
2. The dielectric constant of the organic solvent may influence the equilibrium of the protolytic dissociation. The higher the value of the dielectric constant, the higher the degree of ionization of acids and bases.
3. The acid–base property of the solute, expressed by its pK_a value, may change due to the differentiating effect of many organic solvents.

The latter effect of the three is the most significant, as the dissociation constant, K_a, may change many orders of magnitude for different solvents.

The increased selectivity of separation in organic solvents compared to aqueous systems is due to the fact that the levelling effect of water is eliminated. If