5 Chip technology for micro-separation

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5.1 Introduction

The continuous monitoring of a chemical parameter, usually the concentration of a molecular species, is of central importance in analytical science. It also has commercial significance in chemical production, environmental analysis, and medical/clinical diagnostics [1]. Consequently, modern sensing technology must provide real-time measurements at low analyte concentrations.

Ideally, a molecular "sensor" is highly selective, i.e. it transduces chemical information of *one* species to electronic information, at the exclusion of all other components in a sample matrix (Fig. 1a). In addition, a molecular sensor should possess a large dynamic (concentration) range, be highly reproducible, have a short response time, and be able to operate *in situ*. Many different approaches to the molecular (or chemical) sensor have been investigated [2].

In the real world, chemical analysis methods are at best selective; few if any, are truly specific. Consequently, the discrimination of an analyte from potential interferences is more often than not the key step in an analytical procedure. Accordingly, an alternative approach to molecular recognition is to incorporate a separation step prior to transduction (Fig. 1b). This lessens selectivity requirements, and leads to improved sensitivity (due to an appreciable reduction in background signal). This general strategy can be amalgamated into the concept of the Total (chemical) Analysis System (TAS) [1]. A TAS is a device that periodically transforms chemical information into electronic information. Sampling, sample transport, sample pre-treatment, analyte separation, analyte detection and data storage are automatically performed in a flowing stream. Sample pre-treatment and analyte separation serve to discriminate molecular signals, and consequently lower the selectivity requirements of the detector. This approach to analysis allows for continuous

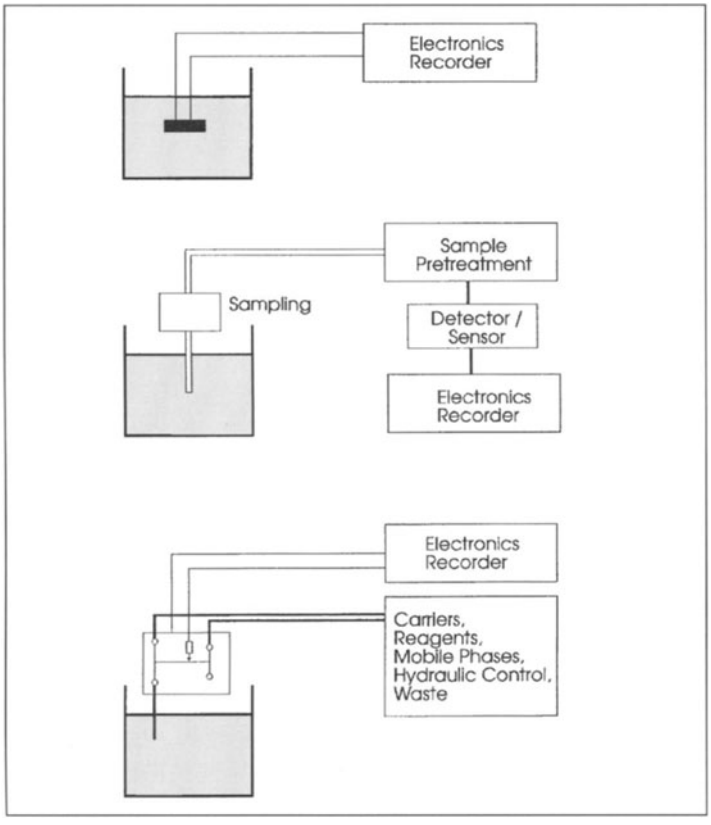

Figure 1 *Schematic diagram of: (a) an ideal chemical sensor, (b) a total chemical analysis system (TAS), and (c) a miniaturised total chemical analysis system (μ-TAS)*

or "real-time" monitoring of molecular systems with time-dependent compositions.

Without question, the most widely used methods for effecting analytical separations are chromatography and electrophoresis. Both have been used with considerable success in TAS research. Each technique exploits the fact that the physical properties of similar molecules are non-identical. In chromatographic separations, the sample matrix is dissolved in a *mobile phase*, which may be a gas, liquid or supercritical fluid. Subsequently, this mobile phase is forced through an immiscible *stationary phase*, which is immobilised on a surface or in a column. The two phases are chosen so that the components of the sample matrix distribute themselves between mobile and

stationary phases to varying degrees. Components which interact strongly with the stationary phase move slowly with the flow of mobile phase, whereas weakly interacting species travel more rapidly. Accordingly, sample components separate into discrete bands that can be analysed quantitatively.

Electrophoresis describes the movement of electrically charged species (ions or molecules) in a conductive, liquid medium under the influence of an applied electric field [3]. Figure 2 illustrates the basic concept. A sample containing a mixture of anions, cations and neutral species is injected at the aniodic end of the system. If an electric field is then applied across the electrolyte, the ions in the sample will tend to migrate through the column at varying rates, and in different directions. The rates and directions of migration depend on the sizes of the ions, and the magnitude and polarity of their charges. For example, cations will migrate toward the negative electrode at a rate that is directly proportional to their charge-to-mass ratio. Separation can then be effected through inherent differences in electrophoretic mobilities. Even though anions will be attracted to the positive electrode (anode), net displacement under normal conditions is toward the cathode. This is due to an electrokinetic phenomenon termed electroosmosis. Electroosmosis occurs as a result of surface charge on capillary walls. This charge causes the formation of an electrical double layer. When an electric field is applied to the system, mobile positive charges within this layer migrate in the direction of the catho-

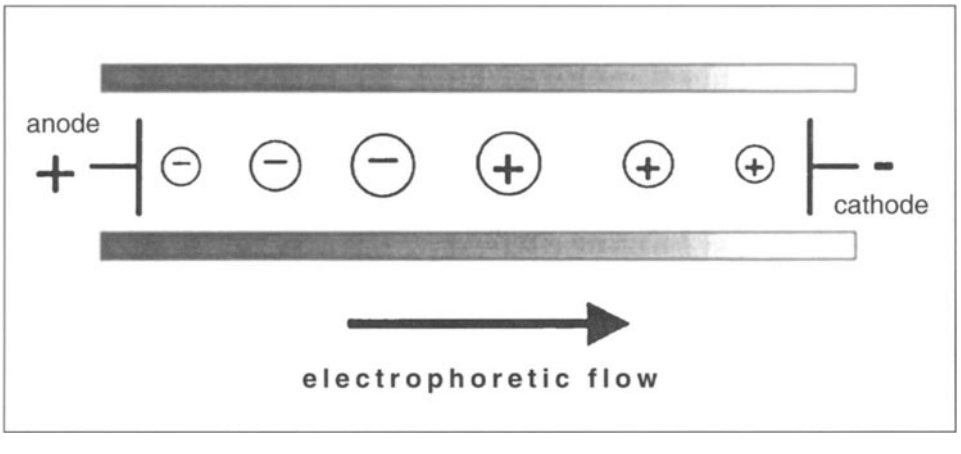

Figure 2 *Schematic representation of capillary electrophoresis. In this situation, sample is introduced at the anode. The circled +'s and –'s describe cationic and anionic species, respectively*

de and drag solvent (electrolyte) molecules along with them. This effect is transmitted throughout the diameter of the capillary, causing a net flow toward the cathode. Detailed descriptions of electroosmotic theory can be found elsewhere [3, 4].

TAS have been applied to a diversity of chemical problems. The main driving force for much research is the need for real-time monitoring and environmental control in industrial processing. For example, TAS able to monitor hazardous substances such as DMS (dimethylsulfate), ECH (epichlorohydrine) and BCME (bis(chloromethyl) ether) have proved extremely useful in the chemical industry [5].

Unfortunately, the TAS approach to chemical analysis is beset by significant problems which hinder its more widespread commercial application. These include slow transport of sample during analysis (especially in the liquid phase), poor separation speed (in the case of electrophoresis and liquid chromatography), excessive consumption of reagents and carrier solutions, and the necessity to fabricate efficient interfaces between the individual components of a TAS.

The concept of *miniaturisation* addresses all of the above problems. Miniaturisation of system dimensions reduces the transport distance between sampling and detection. Furthermore, and more importantly, diffusion and hydrodynamic theories predict higher separation speeds and improved resolution of sample components as channel dimensions diminish [2, 6]. If instrumental dimensions can be made small enough, then analysis times can become comparable to those of traditional chemical sensors (< 500 ms). These miniaturised analysis instruments have been christened μ-TAS (micro-Total (chemical) Analysis Systems) [1]. With sample handing, chemical reactions, sample separation and detection integrated on a *single* device, the μ-TAS resembles a chemical sensor in both size and response. Furthermore, each component function is now under the dynamic control of the user. Figure 1c illustrates the fundamental characteristics of a μ-TAS. Unlike the TAS (Fig. 1b), all sample handling is performed at a location extremely close to the initial sampling.

The following question is often asked: *Is miniaturisation really necessary*? Probably the most obvious gain through miniaturisation is the lowering of sample requirements. Diminished sample consumption permits greater numbers of analyses, which can be significant when working with small amounts of

pharmaceutical and biological material. However, as already noted, a more significant benefit of miniaturisation is the dramatic reduction of analysis times. This reduction allows for the "real-time" monitoring of many molecular systems. Currently, the most obvious need for fast analyses lies in areas such as high-throughput drug screening [7] and genomics [8]. In particular, completion of the Human Genome Project is an enormous task of the highest priority [9]. Unfortunately, current opinion calls for a processing capability of at least 2 orders of magnitude greater than existing DNA analysis methods. In addition, combinatorial chemistry holds the promise of vastly accelerating the rate of drug discovery [10]. The combination of chemical building blocks using parallel, multiple reactions can generate huge numbers of compounds on very short timescales. Subsequently, these combinatorial libraries of compounds need to be rapidly screened to generate new drugs, or explore structure-activity correlations.

Finally, the most significant and exciting possibility afforded by miniaturisation is the creation of highly integrated μ-TAS. These systems will combine all aspects of chemical analysis on a single, integrated device (such as a microchip). Integration will permit automation, which in turn will allow unrivalled reliability, ease of use, and low cost. Yes, miniaturisation is a necessary step in the creation of the ultimate μ-TAS: the *laboratory-on-a-chip*.

The aim of this chapter is to provide a panoramic perspective of the basic philosophies, concepts and current advances in μ-TAS technology. The theoretical aspects of miniaturisation will be introduced, so as to develop a few simple rules for microstructure design. Subsequently, the basic techniques in microstructure fabrication will be outlined, and the use of μ-TAS as tools for microseparation will be discussed. Finally, some novel approaches to detection and component integration will be surveyed. It should be noted that some aspects (such as detection) will be discussed in greater detail elsewhere in this volume.

5.2 Theory of miniaturisation

As already stated, the primary objective of miniaturisation is to enhance analytical performance (i.e. speed of separation, component resolution and

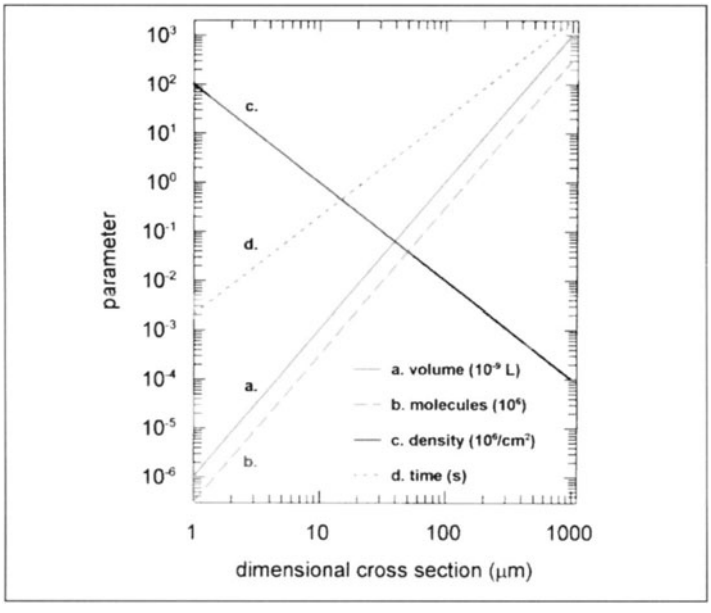

Figure 3 *Scaling of: (a) volume of solution, (b) number of analyte molecules, (c) spatial density, and (d) time to diffusively mix, with dimensional cross section Analysis is performed within a cube of dimension d. A bulk solution concentration of 0.5 nanomolar and a diffusion coefficient of 5 × 10⁻⁶ cm²s⁻¹ are assumed. See text for discussion*

throughput). According to a method described by Ramsey, the scaling of a chemical system can be illustrated simply through reference to Figure 3 [11]. Here, analysis is performed within a detection cube of dimensional cross-section, d (1–1000 μm). The variation of four relevant parameters (probe volume, molecular population of probe volume, spatial density and diffusion time) as a function of d are calculated.

Probe volume and molecular population

It is observed that at the 1 μm dimension our probe volume is approximately 1 fL. For a bulk solution concentration of 5×10^{-10} moldm^{-3} this would mean, on average, less than one target molecule existing at any instant within the volume element. Detection under these conditions is extremely difficult,

although not impossible with modern technology [12]. At the 50 µm scale, the probe volume is now 125 pL and contains approximately 75000 molecules. Molecular recognition, using various protocols, is easily attainable in this regime. At the 1 mm scale, the probe volume is 1 µL and at least 600 million molecules exist in the volume element! According to this rough examination, detection issues imply a normal dimension range of between 10 and 100 µm.

Diffusion time

The ultimate µTAS will perform chemical reactions. On an ultra-small scale these reactions will be mediated by the diffusion of reagent molecules within the reaction chamber. The diffusion of any molecular species is characterised by a specific diffusion coefficient, D (cm^2/s) [13]. In the simplest case of a spherical (or globular) molecule,

$$D = \frac{kT}{6\pi\eta r} \tag{1}$$

where $k = 1.38 \times 10^{-23}$ JK^{-1}, T is the absolute temperature (K), η is the absolute viscosity ($gcm^{-1}s^{-1}$) and r is the hydrodynamic radius. A small molecule (molecular weight *ca.* 500–1000) would be expected to have a diffusion coefficient of approximately 5×10^{-6} cm^2s^{-1}. Under these conditions the molecule will diffuse across a dimension of 1 µm in approximately 2 ms. For a dimension of 50 µm, the molecule will take 5 s, and for a dimension of 1000 µm the diffusion time will increase to 2×10^3 s! This clearly demonstrates that purely diffusive mixing of reagents is only viable for dimensions well below 100 µm.

Scale of integration

It is also of commercial interest to consider the potential scale of integration (device density). This has obvious implications in terms of parallel processing and cost. As can be seen from Figure 3 device density varies as a function of $1/d^2$. Thus for a dimension of 1 µm, 10^8 devices per cm^2 is a theoretical possi-

bility. This decreases to 10^2 devices per cm^2 for a dimension of 1000 µm. Clearly, smaller dimensions are desirable, but can only be achieved using superior fabrication technologies.

The use of capillary electrophoresis (CE) within the µ-TAS format was realised and pioneered by Manz and Harrison in the early 1990s, and it has become the dominant liquid phase separation technique used in the microchip format [14]. Consequently, if we consider how the *parameters of interest* in CE (e.g., number of theoretical plates and Péclet number) vary as a function of the *variables to miniaturised* (space and time), we can see how miniaturisation affects the characteristics of the separation process.

Broadly, a CE system can be defined according to its column length L, inner diameter d, and analysis time t. Time is not directly related to L or d, resulting in one degree of freedom. Table 1 illustrates two possible systems where time is proportional to either L (length) or $d.L$ (cross-sectional area) with power per unit volume and power per unit length as a constant, respectively. Accordingly, it can be seen that all remaining parameters are solely related to L and d.

Table 1 Example of proportionality analysis for capillary electrophoresis. The miniaturisation factors are d and L. Two arbitrarily chosen time dependencies are shown here. The remaining parameters are calculated using the basic definition of d, L and time

Parameter	Symbol	L system	dL system
Capillary diameter	d	d	d
Capillary length	L	L	L
Time	t	L	$d.L$
Linear flow rate	$u = L/t$	constant	$1/d$
Péclet number	$v \propto u.d$	d	constant
Reduced plate height	$h = 2/v$	$1/d$	constant
Number of theoretical plates	$N = L/(d.h)$	L	L/d
Electric field	$E \propto u$	constant	$1/d$
Applied voltage	$U = E.L$	L	L/d
Electric current	$I \propto U.d^2/L$	d^2	d
Power/volume	$P_v \propto (I.U)/(d^2.L)$	constant	$1/d^2$
Power/length	$P_l \propto (I.U)/L$	d^2	constant
Temperature difference	$\Delta T \propto I^2$	d^4	d^2

The L system is characterised by a timescale that is proportional to L. Thus, if L is reduced by a factor of two, the retention time will also be reduced by a factor of two (assuming a constant electric field). A concomitant decrease in the number of theoretical plates is noted. In the $d.L$ system the timescale varies with the cross-sectional area. This means that the Péclet number, reduced plate height and power per unit length remain constant. Importantly, it can now be seen that an improvement in both analysis time and separation performance can be achieved by simply reducing the ratio of d to L. For example, if the diameter of a capillary is reduced from 100 to 10 µm, whilst the capillary length is reduced from 10 to 2 cm, the number of theoretical plates would increase by a factor of two, in 1/50th of the retention time! This system has been experimentally shown to exist [6].

A thorough analysis of miniaturisation theory by Manz and co-workers yields some simple rules for the design of electrophoretic microstructures [2, 15].

Separation efficiency

(a) The *absolute voltage* drop between the points of injection and detection defines the maximum number of theoretical plates (N), and the corresponding resolution of two neighbouring peaks ($\sqrt{N}$). The higher the applied electric field the higher N is.
(b) To minimise Joule heating and heat transfer across the capillary, a power of 1 W/m should not be exceeded.
(c) When separating species of different charge 10^2 theoretical plates is sufficient. When separating species of similar or identical charge 10^5 theoretical plates are necessary to give satisfactory resolution.

Consequently, the maximum number of theoretical plates (N_{max}) subsequent to miniaturisation is defined according to the following proportionality,

$$N_{max} \propto \frac{L}{d} \tag{2}$$

In other words, if both L and d are reduced by the same fraction, there is no loss in analytical performance. Furthermore, N_{max} will be increased by either decreasing d or increasing L.

Analysis time

(a) The length of the capillary dictates the analysis time.
(b) The efficiency of the separation column will only be discernible if the contributions of injection and detection processes are small with respect to both the volume or length of the column.

Accordingly, the minimum analysis time is proportional to the product of the capillary length and internal diameter, i.e.

$$t_{\min} \propto L \cdot d \tag{3}$$

Hence, a reduction in analysis time can be achieved by either reducing L or d. However, it must be noted that the only way to achieve a reduction in analysis time whilst maintaining the efficiency of the separation will be to reduce L and d by the same factor.

5.3 Fabrication and structure of μ-TAS

The stringent demands of μ-TAS have meant that conventional manufacturing methodologies are not applicable to miniaturised systems. The fabrication of flow manifolds, reactors, electrodes, sieves etc. on a micron scale is a non-trivial task. Consequently, the standard form of a μ-TAS is a planar, chip created using *micromachining* techniques. Micromachining can be defined simply as "the sculpturing of silicon and silicon compatible materials" to produce devices having no direct electrical function [16]. Micromachining methods were conceived as early as the 1850s through the work of Grove, Edison and Fleming [17]. Nevertheless, the real applications of micromachining technology were only realised a century later when the microelectronic revolution began. Over the next three decades a diversity of silicon-based sensors and actuators were developed, at an ever increasing rate and scale of integration. Today, techniques such as reactive ion etching [18] and LIGA [19] permit even more elaborate 3D microstructures to be made. The creation of sophisticated microelectromechanical systems (MEMS) such as micro-accelerometers, blood pressure sensors, infusion pumps and ultra-high reso-

lution inkjet nozzles are now commonplace [20, 21, 22]. The μ-TAS can be considered a distinct subcategory of the MEMS concept, i.e. a microsystem for chemical analysis.

One of the primary strengths of micromachining is the reproducibility achieved in manufacturing. Typically, each silicon wafer will eventually yield hundreds or thousands of individual and identical "devices". More specifically, micromachining brings together the generic methods of photolithography, special etching, film deposition and bonding to create three-dimensional microstructures [23]. The fundamental idea is to sequentially superimpose two-dimensional patterns onto a substrate surface. Each pattern defines an area to be etched or an area onto which a new material can be deposited. The final, "three dimensional" structure is simply the result of these additive or subtractive steps.

A variety of substrate materials can be used in the manufacture of μ-TAS. These include silicon, glass, quartz, metals, plastics and ceramics. To date silicon, quartz and glass are the substrate materials of choice (primarily due to chemical, electrical and optical properties). However, it is noted that recent developments in polymer micromachining technology have enabled the construction of novel, moulded microstructures which offer high analytical performance at low cost [24, 25]. Since, detailed discussions of micromachining technologies are given elsewhere in this volume, only a cursory survey of these methods will be provided in the current chapter.

Photolithography

Photolithography is universally used to define the regions where subtractive and additive processes will act. The basic procedure starts with the deposition of a durable, photosensitive polymer (photoresist) onto the substrate surface. This is normally achieved by dropping a solution of the photoresist onto the surface, whilst spinning the substrate at many thousand rpm. This results in the formation of a uniform, photoresist layer a few microns thick. The coated surface is baked, to drive off any excess solvent, and then exposed to UV radiation though a chrome mask. Subsequent development in an organic solvent allows the removal of exposed portions of photoresist,

leaving a polymerised resist pattern with high chemical resistance. This resist mask is then used to define one layer of the microstructure being fabricated.

Etching

The most simple subtractive process is etching. Etching allows the two-dimensional photoresist pattern to be transferred to the substrate material, through use of "wet" or "dry" protocols. Wet etching involves the use of aqueous etchants. These etchants are differentiated on the basis of whether they act in an isotropic or anisotropic manner. Examples include HF, HNO_3 (isotropic etchants), KOH and tetramethyl ammonium hydroxide (anisotropic etchants). Etchants act by oxidizing silicon to silicates [23].

Isotropic and anisotropic "dry" etch protocols exist for silicon, dielectric materials, metals and organics. These methods generally involve the use of partially or fully ionized gas plasmas to effect the etch. Extremely high degrees of anisotropy are possible using plasma-based techniques, allowing the fabrication high aspect-ratio microstructures.

Film deposition

This term encapsulates a wide variety of additive processes that enable microstructures to be built on the substrate surface. For silicon, the simplest additive process is thermal oxidation to generate a SiO_2 layer. More correctly this is a reactive process since temperatures in excess of 900°C are normally necessary. For subsequent deposition of secondary materials a variety of low-temperature processes can be used. These include spin-coating, physical vapour deposition (PVD), chemical vapour deposition (CVD), low pressure CVD, plasma enhanced CVD (used when low temperature processing is desired) and sputter deposition [23, 26, 27]. A huge number of materials can be uniformly deposited using these techniques. Table 2, lists some examples of these materials and the processes that enable their deposition. Depending on the material and the deposition process used, film thicknesses of a few Angstroms to many microns can be obtained.

Table 2 Common additive processes used in micromachining

	Thermal	CVD	PECVD	Sputter	Electro-plating	Spincasting/ screenprinting
	> 900°C	600–1000°C	100–600°C	< 300°C	ambient	ambient
SiO_2	x	x	x	x		
Glass				x		x
Single crystal silicon		x				
Metals				x	x	x
Polysilicon		x				
Amorphous silicon			x	x		
Polymers		x	x			x

Bonding

This is the final stage in fabrication of a μ-TAS. Bonding simply refers to the assembly of the substrate materials, e.g., silicon-to-silicon, glass-to-silicon, silicon-to-oxide and glass-to-glass. Aniodic bonding is commonly used to bond glass and silicon substrates. This procedure utilises electrostatic attraction to form covalent bonds between the surface atoms of the glass and the silicon. For glass or quartz, thermal bonding (450°–900°C) provides the simplest way to assemble the substrate materials. Furthermore, the planarity of the substrate surfaces generally means that bonding is a simple and efficient final step in the manufacture of μ-TAS [2].

Figure 4 illustrates a simple one-mask micromachining procedure. The process begins with thermal oxidation of the silicon to generate a surface oxide layer. A photoresist film is then deposited on top of the oxide layer. Photolithography and subsequent chemical development define the region to be etched. The first etch step removes the oxide layer. Subsequent etching of the bulk substrate yields the desired channel. If further structural definition is required, additional processes would augment the sequence shown in Figure 4 (e.g., CVD of metal films). The final step in creation of the complete microstructure is bonding to a top plate. This allows encapsulation of the open channel. An example of a μ-TAS is shown in Figure 5.

The procedure illustrated in Figure 4 describes the manufacture of the simplest kind of μ-TAS; an enclosed channel on a microchip. The need for

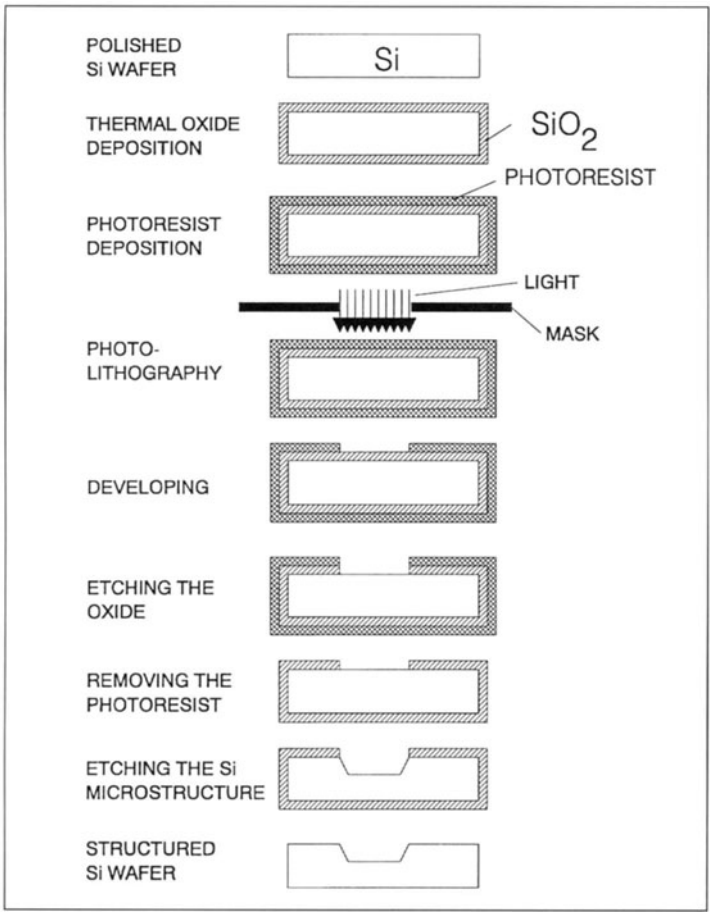

Figure 4 *Process steps of a standard one-mask micromachining procedure for etching a single channel into a substrate material*

additional components (for flow handling and detection) is obvious, but instantly adds an extra dimension to the level of micromachining technology necessary. The following paragraphs briefly outline some of these components.

Devices for handling liquids and gases are key elements in μ-TAS. Research on silicon micropumps was pioneered by Wallmark and Smits in the early 1980s [28]. Since then, a diversity of micropumps and microvalves have been developed. Most micropumps are of the reciprocating kind (i.e. diaphragm pumps) [29, 30, 31]. These pumps in their most basic form consist of a dis-

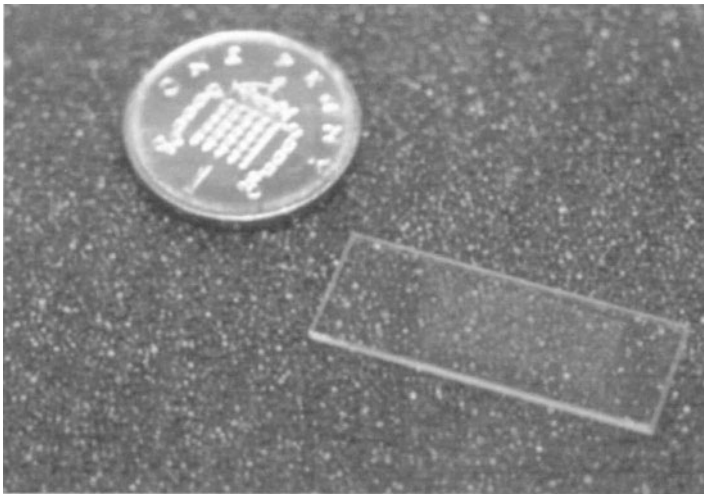

Figure 5 *Scanning electron micrograph of part of a continuous flow PcR microchip*

placement unit and two passive check valves. Almost all liquids can be manipulated using diaphragm pumps, with achievable flow rates of nanoliters per minute up to a few milliliters per minute. Several physical principles have been applied to the actuation of micro diaphragm pumps. These include piezoelectric actuation [32], electrostatic actuation [33], electromagnetic actuation [34], bubble actuation [35] and thermopneumatic actuation [35]. To date no commercial fluid systems incorporate micropumps. This is primarily due to problems in pump priming, particulate pollution and the fact that construction is both complex and expensive [36]. In addition, although flow-rate stabilities and lifetimes are reasonably good, most pumps are not suited to operate in high pressure situations. More recent research has led to the development of pumps (with high pressure check valves) with improved forward-to-reverse flow behaviour at pressures above 1 atm [37].

Valves are one of the most fundamental components of a μ-TAS and need to be designed with the operation of a particular chemical system in mind. Fortunately, under many circumstances (e.g., microelectrophoresis) the need for physical valves is obviated through the use of electrokinetic switching [38]. Nevertheless, in more sophisticated procedures efficient and robust microvalves are necessary.

To date, most microvalves have been developed for handling gases [33, 34, 39], although many can now handle both liquids and gases [32, 40]. These valves are actuated using a variety of mechanisms [41]. System aspects such as dead volume, leakage, size and simplicity play a dominant role in design. Microvalves can work discretely or in combination. By coupling two microvalves with a membrane, a simple micropump can be easily formed. When the membrane is contacted with the glass surface analyte cannot move between the inlet and outlet. However, if the membrane is pulled away from the surface, analyte can flow freely between channels. In this case actuation is based on pneumatics [42]. In addition Esashi and co-workers have reported several actuated, three-way valves for gases and liquids, as well as miniaturised mass flow controllers for gases [32].

Temperature sensors can be easily fabricated on most substrates. The most obvious form of a temperature sensor is a thin film thermocouple, where sensing is based on thermoelectric phenomena. Thermocouples of this kind can be made using evaporative deposition techniques [43].

The potentials of μ-TAS will only be realised if "real samples" can be effectively handled and analysed. Samples of this kind (e.g., blood, semen and saliva) will almost certainly contain particulates and gas bubbles, which pollute the microsystem both chemically and physically. Particulates cause the most serious problems. For example, any particulate matter in a microchannel may cause a local blockage or render delicate micropumps and microvalves useless. Consequently, the need for microsieves, filters and bubble separators is apparent. These can be made using fairly simple micromachining technologies [44, 45]. Pore diameters can be precisely defined to match particular species present in sample. Sieves will remove both particulates and bubbles.

The fabrication of mixers/reaction chambers is a non-trivial process. The downsizing of the fluidic process to the femtoliter-nanoliter range leads to Reynolds numbers which are significantly smaller than values characteristic for the onset of turbulence. Consequently, novel mixing protocols must be developed, an example of which has been presented by Miyake [46]. They propose a situation where a fluid is forced through a matrix of holes perpendicular to the flow direction of a second fluid. The fluid that is forced through the "sieve" emerges as micron-sized globules. This leads to an increase in the contact area of the fluids, yielding efficient mixing of the two fluids. More recently, Larsen and co-workers have demonstrated a novel static mixer for

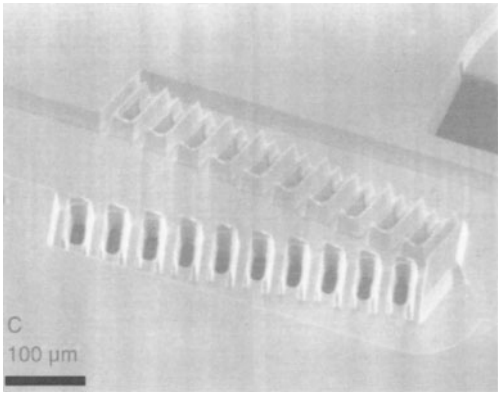

Figure 6 *Scanning electron micrograph of a novel mixer for multilayer lamination (as described in [47])*

chemical and biochemical reactions [47]. Rapid mixing is achieved by multilayer lamination of two liquids in a single microchannel. Figure 6 is an SEM image of the silicon mixer with laminated flows. The advantage of this design stems from the robustness of the mixer to both particulate and bubble pollution.

5.4 Chemical analysis using µ-TAS

The original application of microsystem technology to chemical analyses occurred nearly two decades ago, when Terry and co-workers created an integrated gas chromatograph on a 50 mm silicon wafer [48]. Unfortunately, this seminal piece of work received limited attention at the time; primarily due to performance issues. Nevertheless, in the mid to late 1980s several research groups realised the potentials of miniaturisation in chemical analysis, and applied the concepts of micromachining to liquid phase separation techniques. This was in large part due to the advent of capillary electrophoresis as a dominant analytical tool [3].

Early research was pioneered by Manz and colleagues initially at Hitachi Ltd. [49] and subsequently at Ciba-Geigy [50], where the term µ-TAS was christened. Since then, a number of laboratories world-wide have developed the µ-TAS concept and applied it to a variety of chemical systems. As stated previously, microfabricated devices have been demonstrated for a diversity of chromatographic and electrophoretic separation methods. These µ-TAS have

analytical performances equivalent to, or in excess of their conventional counterparts. This section summarises much of this research, and serves to highlight the diversity of μ-TAS.

Capillary electrophoresis

Capillary electrophoresis (CE) is a relatively new technique for the separation and analysis of chemical species. Since its inception in the early 1980s, its use in the chemical and more significantly, the biological sciences has grown at an exponential rate [51]. CE complements the traditional separation techniques of high-performance liquid chromatography (HPLC), gas chromatography (GC) and slab gel electrophoresis. Many analyses currently utilising HPLC or slab gel electrophoresis will probably convert in time to CE, due to its high efficiency and high throughput. In addition, CE sample requirements are low and capillaries are relatively cheap and long-lasting. As noted previously, CE separations occur due to inherent differences in the electrophoretic mobilities of molecular species. Furthermore, since electroosmotic flow (EOF) is normally in excess of electrophoretic migration, all sample components move in the same direction (but at differing rates). Consequently, EOF can be used to manipulate sample within the capillary.

CE is perfectly suited for application within the μ-TAS framework. This statement can be demonstrated by reference to some fundamental equations. For an ideal CE system, the separation efficiency can be defined in terms of the number of theoretical plates N, i.e.

$$N = \frac{\mu \cdot V}{2D} \tag{4}$$

Here, D is the specific diffusion coefficient (cm^2/s), μ is the electrophoretic mobility (cm^2/Vs) and V the total applied voltage. N is proportional to the applied voltage, regardless of the capillary dimensions. In addition, the time for a zone to migrate a distance L is given by Eq. 5.

$$t = \frac{L^2}{\mu \cdot V} \tag{5}$$

Consequently, it can be seen that high voltages and short capillary lengths generate fast, high efficiency separations. For example, if the length of a capillary is reduced by a factor of two, and the applied electric field doubled, the efficiency of the separation will increase by a factor of two, in 1/8th of the retention time. It should be noted that high applied electric fields however introduce the additional problem of Joule heating. This effect must be offset by reducing the internal diameter of the capillary. Micromachining technology allows the creation of channels which are short in length and have small cross-section dimensions. Thus, the μ-TAS approach should theoretically yield ultra-fast, ultra-high efficiency electrophoretic separations.

Figure 7(a) depicts the layout of a glass chip for CE with integrated sample injection. The separation channel is 50 μm wide and approximately 12 μm deep. Figure 7(b) is an electron micrograph of the region where injection and separation channels intersect. As stated, the primary method of sample transport on a CE chip is electrokinetic pumping (a combination of electroosmosis and electrophoresis). Significantly, sample transport, sample injection and other handling steps can be performed and controlled by the selective application of electric potentials across channels. In contrast, approaches using micropumps and valves suffer from high back pressures, and are generally not suited to the delivery of very small dose volumes [52].

Application of an electric potential between reservoirs 1 (sample) and 4 (injection waste) causes the geometrically defined injection volume ("double-Tee" injection volume ~ 90 pL) to be filled by electrokinetic migration of sample. Once loading is complete, application of a high potential between reservoirs 2 (buffer) and 5 (waste) drives the injected sample plug into the separation channel, and effects the electrophoretic separation of sample components. Figure 8 illustrates an electropherogram obtained using this protocol. A mixture of 6 FITC-labelled amino acids; arginine (Arg), glutamine (Glu), phenylalanine (Phe), asparagine (Asp), serine (Ser) and glycine (Gly) are separated on a CE chip within 14 s. Plate numbers range from 5800 to 160000 for only 20 amol of injected sample. In addition, shorter analysis times were achieved by reducing the separation length (at the expense of resolution) [53]. Due to the simplicity of this generic approach, i.e. microfabricated, interconnected channels on a planar substrate, CE chips have been applied to a diversity of chemical systems. Inspection of the literature illustrates that the vast majority of microchip separation research has centered on electropho-

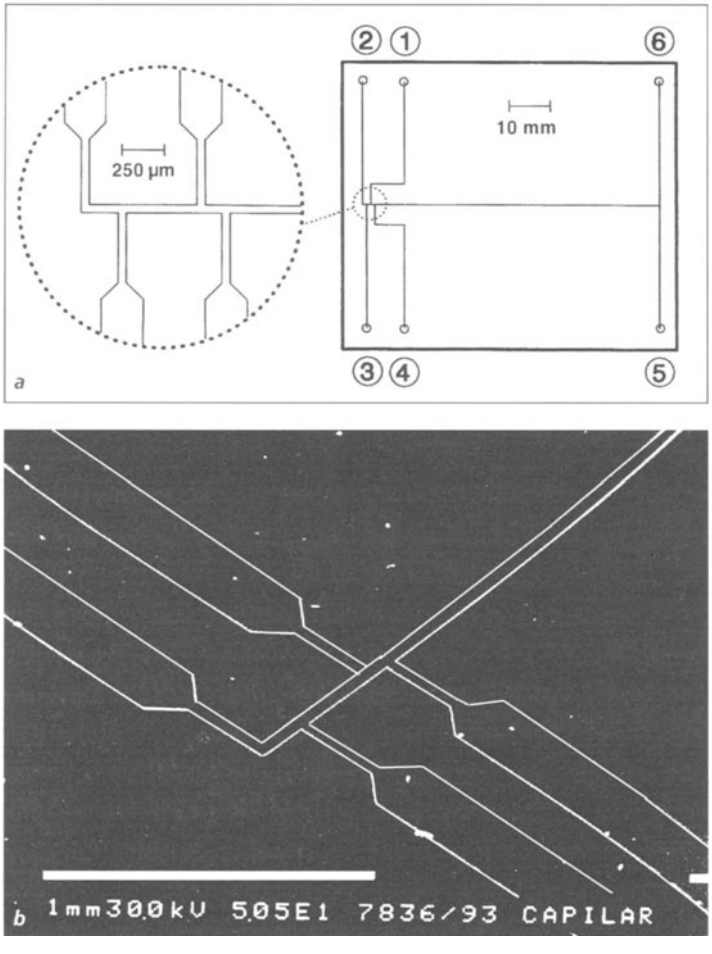

Figure 7 *(a) Layout of glass CE microchip with integrated sample injector. Channel cross-sections: 50 × 12 µm (tin channels) and 250 × 12 µm (wide channels). See text for description of sample manipulation. (b) Scanning electron micrograph of region where injection and separation channels intersect*

retic and electroosmotic mechanisms. The composition of this review will reflect that fact.

The feasibility of using electroosmotic pumping and electrophoretic separation within a planar, glass chip was first demonstrated by Manz and Harrison in late 1991 [54, 55]. Glass CE chips were used to separate a mixture of two fluorescent dyes (calcein and fluorescein), with efficiencies approaching those obtained from conventional fused silica capillaries. Reduction of channel

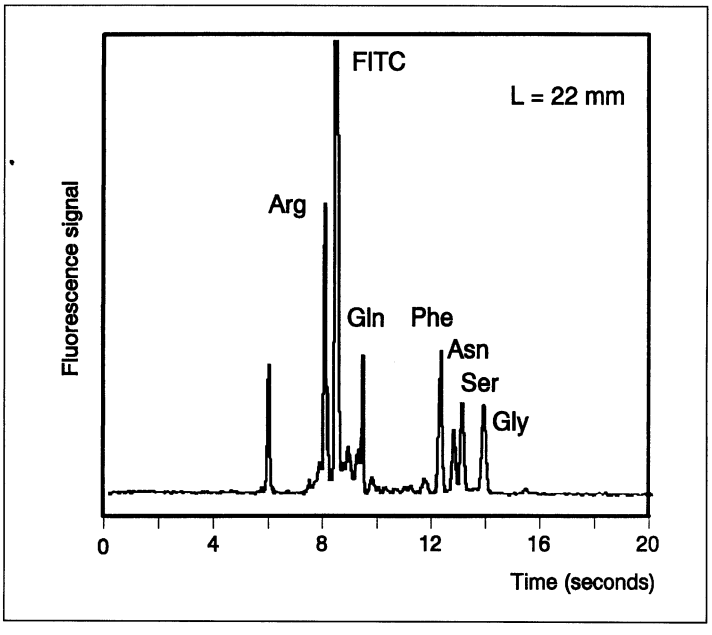

Figure 8 *Electropherogram of a mixture of six FITC-labelled amino acids recorded at a separation length of 22 mm, and an electric field strength of 1060 V/cm. Formal concentration of each amino acid is 10 μM. Solutions were made in a pH 9.0 buffer (20 mM boric acid – 100 mM Tris)*

lengths allowed ultra-high speed separations of fluorescein dye derivatives. For instance, fluorescein and fluorescein sulphonate dyes were separated in a channel 75 mm long within 4 s of injection [56]. Performance evaluation demonstrated that electric fields of over 10^3 V/cm could be applied to the glass substrates without apparent dielectric breakdown [57]. This work was swiftly followed by the application of CE chips to more interesting media. As noted, Figure 8 illustrates a fast and efficient separation of amino acids on a glass microchip. Analysis times for this system ranged from a few seconds to a few tens of seconds, and plate numbers of up to 160000 were realised. In addition, repetitive sample injection and separation cycles yielded highly reproducible migration times and peak areas. These results were significant in that they demonstrated the feasibility of automated, quasi-continuous, on-line monitoring of chemical species [53, 58]. Studies at about the same time indicated that the use of silicon as a possible substrate material was not desirable. Insulation characteristics placed limitations on the size of the applied electric fields, and thus impaired separation efficiencies [59].

In 1994, a number of additional research groups began to apply micromachining techniques to CE. Ramsey and co-workers at the *Oak Ridge National Laboratory* fabricated glass microchips similar in concept to those previously described. Serpentine column geometries allowed channels 165 mm in length to be confined within a 10 mm × 10 mm area. Fast, efficient separations of rhodamine dyes, with minimal bandbroadening effects, were demonstrated [60]. This work was soon followed by an elegant demonstration of ultra-fast CE on a glass microchip. Fluorescein and Rhodamine B mixtures were separated along a microfabricated channel using fluorescence detection (Fig. 9) [61]. At an electric field strength of 1.5 kV/cm, analytes are resolved in less than 1.6 s for an 11.1 mm separation length, in less than 260 ms for a 1.6 mm separation length, and in less than 150 ms for a 900 µm separation length! These results established the suitability of microchip CE devices in sensor applications.

A fundamental study of EOF in microfabricated columns by Seiler et al. deemed channels to behave in an analogous way to resistors (in terms of their electrical characteristics). Accordingly, the potential at any point within the channel network can be estimated using *Kirchoff's Rules* (assuming the solution resistivities are known). Use of this concept enables the direction and magnitude of sample flow to be precisely calculated, thus ensuring a totally valveless fluidic system [38, 62]. Conversely, Jacobsen et al. minimised EOF by covalently bonding polyacrylamide to the channel walls of fused silica CE chips [63]. The reduction of EOF leads to an enhancement in the separation efficiency.

We have seen that miniaturisation of the analytical process yields improvements in efficiency and speed. In addition, sample and reagent requirements are reduced. However, perhaps the most exciting possibility afforded by miniaturisation is the creation of highly integrated µ-TAS. Many laboratory-based procedures require sample manipulation prior to the measurement process, and some analyses are fully automated to avoid operator bias. Consequently, the evolution of µ-TAS must encompass the whole analytical process; not just the separation. The first attempt to address the idea of integration involved the construction of a *postcolumn* reactor on a glass microchip [64]. Two amino acids were separated in a microfabricated channel. Each component was then reacted with *o*-phthaldialdehyde, as it passed through a mixing tee, to form a fluorescent moiety. These are subsequently detected

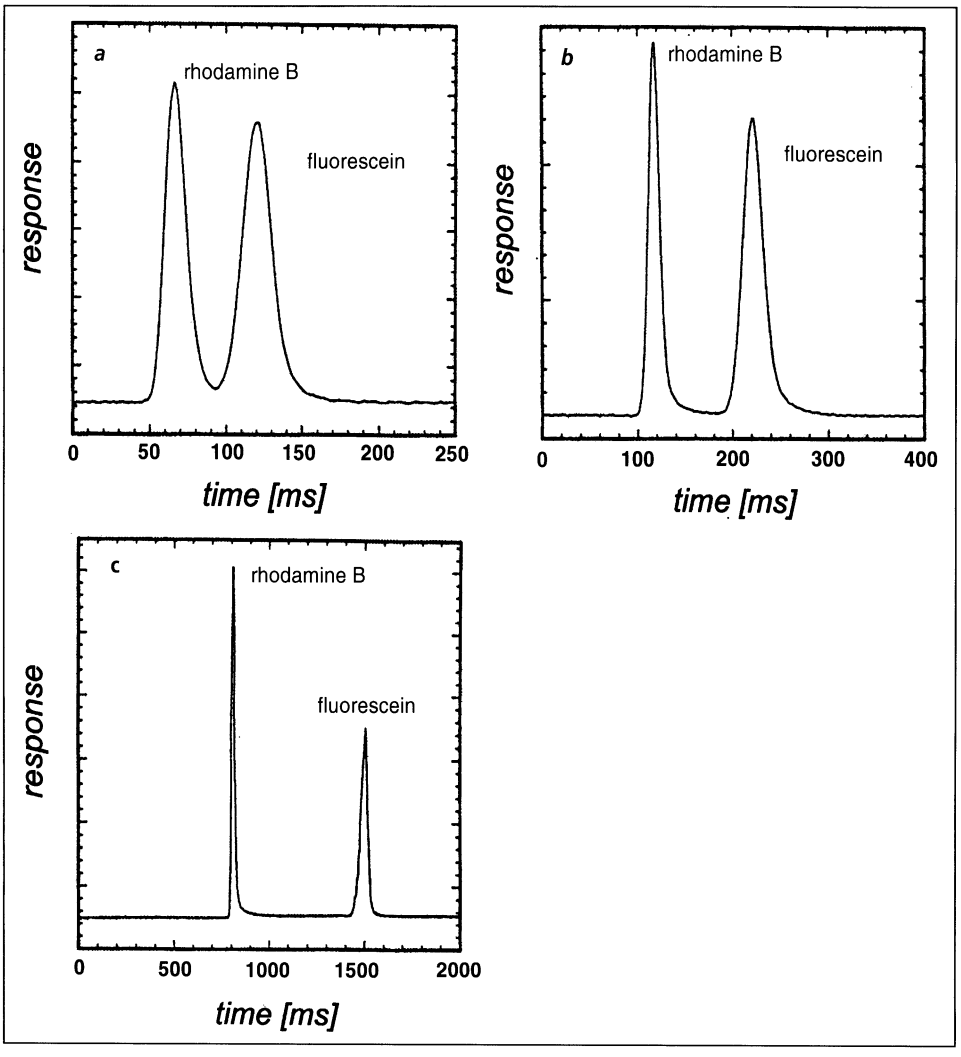

Figure 9 *Electropherograms of rhodamine B and fluorescein with a separation field strength of 1.5 kV/cm and a separation length of: (a) 0.9 mm, (b) 1.6 mm, and (c) 11.1 mm*

using laser induced fluorescence. This work was soon followed by the first example of an on-line *precolumn* reaction and CE on a glass microchip [65]. Amino acids (arginine and glycine) were reacted with *o*-phthaldialdehyde in a 1 nL reaction chamber (96 µm × 6 µm × 2000 µm) at room temperature, to yield fluorescent products. The product mixture is then electrokinetically injected into the separation channel, and electrophoresed. Species were detected using confocal laser induced fluorescence. Highly reproducible separations were obtained within a few seconds of injection.

Since the microfabrication of intersecting channels creates no intermediate dead volumes, planar CE chips lend themselves to applications which are impractical using conventional, fused silica capillaries. An elegant example of which is synchronised cyclic capillary electrophoresis (SCCE). This technique (established by Burggraf and Manz) permits extremely efficient separations of molecular species at low applied voltages [66]. Detailed descriptions of SCCE are given elsewhere [66]. Nevertheless, the principle behind SCCE can be clearly illustrated by reference to Figure 10. The procedure allows for the elimination of *too slow* or *too fast* components, and concurrently improves the resolution of the remaining components [67, 68]. The applied voltages are rotated by 90 degrees relative to a layout of four-fold axial symmetry, and voltage switching is synchronised to a particular component. During a single cycle, species migrate in a field that corresponds to twice the applied voltage, thus yielding higher plate numbers (*cf.* Eq. 4). Figure 11 illustrates an electropherogram obtained using a SCCE chip of channel circumference 80 mm. It can be seen that using a separation length of 20 mm, six fluorescein isothiocyanate labelled amino acids are separable within a few seconds. In addition, it is noted that any fully integrated μ-TAS will need to isolate products sub-

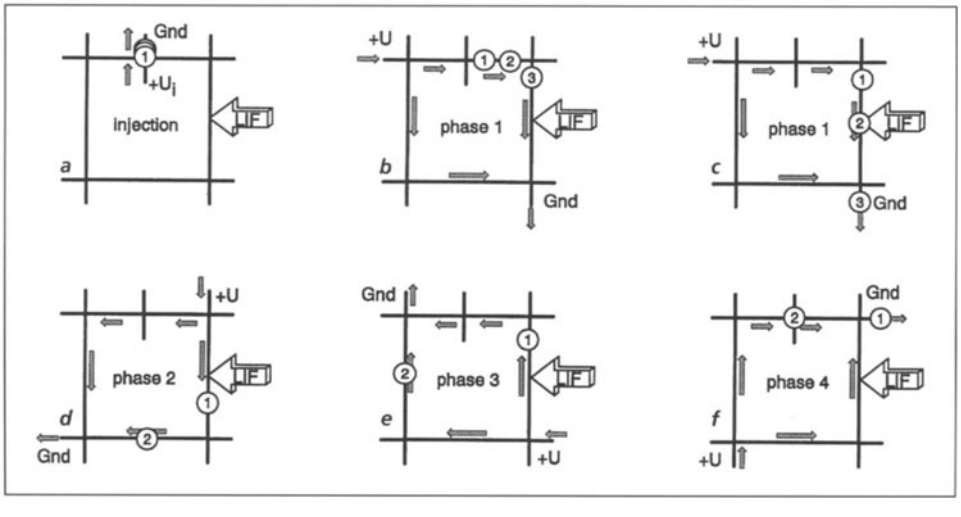

Figure 10 *Principle behind synchronized cyclic capillary electrophoresis (SCCE). Three sample components are represented by circled numbers (① ② ③). The voltage switching procedure is synchronized to component 2: (a) injection phase, (b) during phase 1, (c) at the end of phase 1, (d) phase 2, (e) phase 3, (f) end of cycle*

sequent to reaction and separation stages. SCCE provides a well defined mechanism for doing this, even for complex mixtures.

We have seen that miniaturisation of the electrophoretic process leads to huge gains in analysis time and efficiency [61]. In addition, the nature of the microfabrication process allows the manufacture of many identical devices in a single batch (hundreds to thousands). Consequently, the combination of microfabricated electrophoresis channels with the ability to analyse multiple channels in parallel leads to enormous gains in information density and sample throughput. As noted previously, this has the most significant implications in the areas of combinatorial chemistry [10], drug screening and genomics [9].

The human genetic blueprint – the human genome – consists of an estimated 100000 genes, which encode information in DNA for making and maintaining human life. These genes are distributed among 23 pairs of chromo-

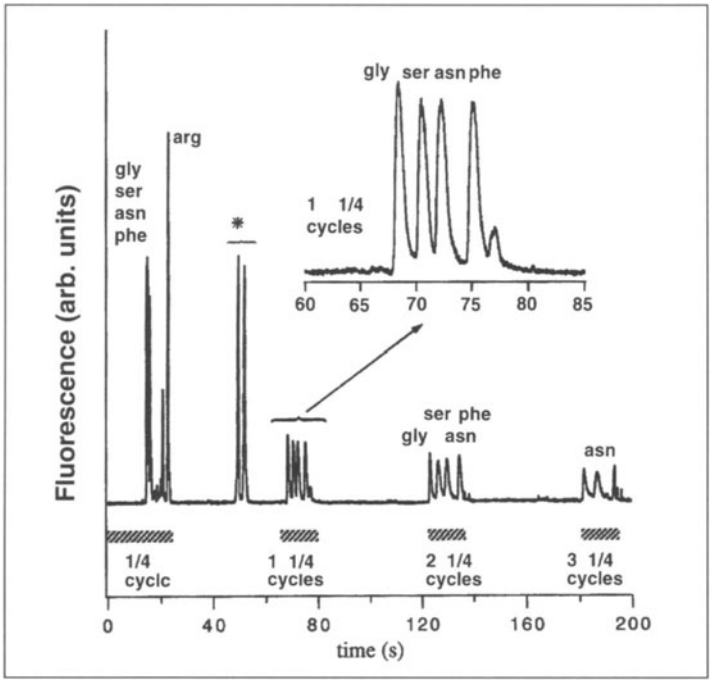

Figure 11 *Synchronised cyclic CE separation of FITC-labelled amino acids. Synchronization time interval is 14.4 s, with the hatched bars indicating times during which zones of interest are detected. The peak marked with an asterisk represents samples that are redetected due to channel switching*

somes, each containing a long DNA molecule. There are a total of 3 billion bases in the complete human genome. CE has proved to be a powerful technique for DNA analysis, and has been applied to restriction fragment sizing, PCR product analysis and DNA sequencing [51]. Although considerably faster than conventional slab gel separations, the current demands of the Human Genome Project require increased processing capabilities [8]. The ability to separate DNA on microfabricated CE arrays would clearly meet that need, and establish the feasibility of integrated DNA analysis devices. The first examples of DNA separations on a microfabricated CE devices were presented in 1994. Woolley and Mathies performed high resolution electrophoretic separations of ϕX174 *Hae III* DNA restriction fragments (72–1353 base pairs) in microfabricated channels filled with a hydroxyethylcellulose sieving matrix [69]. DNA fragments were labelled with dye in the running buffer, and detected using a laser-excited, confocal fluorescence system. Electrophoresis in 35-mm-long channels allowed complete separation of all fragments in less than 2 min. This work clearly established protocols for high-speed, high-throughput DNA analysis. Concurrently, Effenhauser et al. demonstrated fast separations of synthetic phosphorothioate oligonucleotides (10–25 base pairs) on similar glass microchips [70]. Application of electric fields of up to 2.3 kV/cm resulted in size separation of single-stranded oligonucleotides in less than 45 s. Both these studies suggested that with sensitivity and resolution improvements DNA sequencing on chips was possible.

Within a year, Woolley and Mathies published the first demonstration of DNA separations with single base pair resolution on microfabricated CE glass chips [71]. Electrophoresis was performed in 50×8 µm cross-section channels, using a denaturing 9% *T*, 0% *C* polyacrylamide sieving matrix. Fragment ladders were fluorescently labelled using novel energy transfer dye-labelled sequencing primers [72]. Sequencing extension fragments were separated to 433 bases within 600 s using a one-colour detection protocol and a separation distance of 35 mm. Using a four-colour chemistry, DNA sequencing (with 97% accuracy) to 150 base pairs was achieved in only 540 s. Although improvements in resolution and run length are necessary, the authors postulate that CE array chips could realistically yield a raw DNA sequencing rate of 4×10^4 bases/hour per chip! Very recent work by Woolley et al. has established the feasibility of high speed DNA genotyping using CE array

chips. Both restriction fragment sizing and genotyping of the HLA-H gene have been performed in 12 parallel channels within 160 s [73]. In addition, Effenhauser and co-workers have developed moulded, silicone elastomer microchips for DNA fragment analysis. PDMS-microchip separations of ϕX174 *Hae III* DNA restriction fragments stained with an intercalating fluorescent dye are complete in a few minutes. Improvements in detection protocols have also allowed the detection of single λ-DNA molecules under electrophoretically controlled flow conditions [24]. It should be noted that the manufacturing cost of a silicone chip is approximately $ 0.7. This low price should justify a single-use, disposable approach to μ-TAS devices in areas such as medical diagnostics and screening.

One of the eventual requirements of a fully integrated μ-TAS will be the ability to isolate a product subsequent to reaction and separation stages. In conventional CE, this is a non-trivial task [74, 75]. However, this assignment becomes far simpler when applied to microfabricated networks of intersecting channels. A refined study by Effenhauser et al. used a very simple channel network for selective isolation of fraction zones after fast sizing of phosphorothioate oligonucleotides [76]. Withdrawal of sample was effected by automated switching of applied potentials. Importantly, the authors demonstrated that a single sample component, originally injected into a 90 pL volume prior to separation, was still confined to a volume of approximately 300 pL after isolation. This degree of sample control has particular relevance for future developments the creation of μ-TAS for biochemical analysis.

To further develop these microsystems towards a complete μ-TAS, some degree of sample pre-treatment will be required. This may involve filtration or sieving (as discussed previously), isolation of components, or even complex chemical reactions. Raymond and co-workers have applied the technique of free-flow electrophoresis (FFE) to the problem of sample pre-treatment using a silicon microchip device [77]. The general principle of FFE is illustrated in Figure 12. A narrow sample stream is fed into a carrier solution which flows perpendicular to an applied electric field. Consequently, charged species are deflected from the direction of flow at an angle determined by a combination of the carrier flow velocity and the electrophoretic mobility of the component [77, 78]. After separation, the individual sample components can be collected as they leave the separation bed. This approach is advantageous since isolation and subsequent delivery to the analysis system can be done in a con-

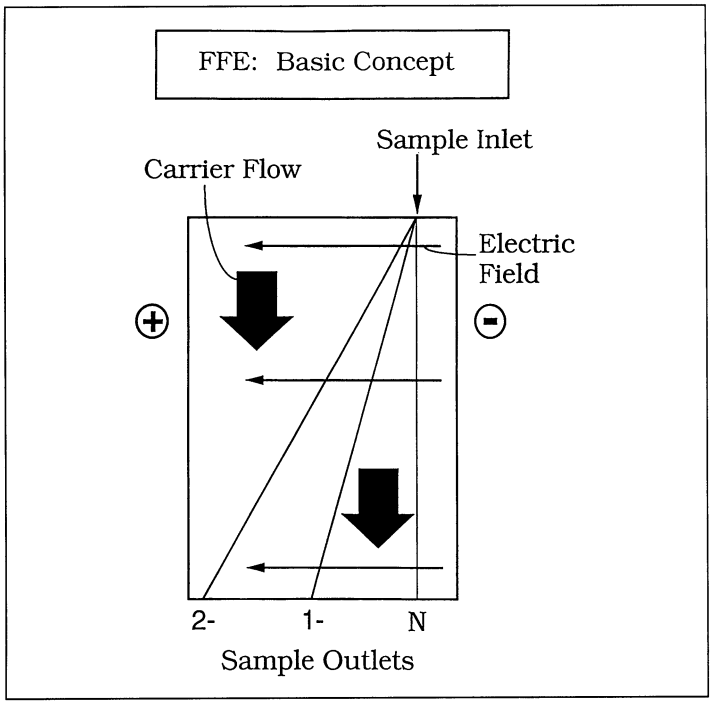

Figure 12 *Principle behind free-flow electrophoresis. The positions N, −1, −2 illustrate where species of formal charge 0, −1, −2 respectively exit the microstructure*

tinuous manner. Figure 13(a) shows a schematic of the silicon FFE chip. Carrier is introduced through two inlets on either side of the sample inlet. Once sample is transported into the separation bed (10 mm × 50 mm × 50 µm) electrophoresis occurs at right angles to the direction of carrier flow. Figure 13(b) is a three-dimensional view of the inlet region. Using this silicon chip it is possible to achieve a continuous separation of small ions according to their charge. Figure 14 illustrates the continuous separation of three labelled amino acids (lysine, glutamine and glutamic acid). An applied potential of 50 V gave baseline resolution of ions differing by one charge unit in 2–5 min. The application of µ-FFE to the continuous separation of high molecular weight compounds has also been demonstrated [79]. Using a silicon device with a separation bed volume of 25 µL, the continuous separation of human serum albumin (HSA), bradykinin and ribonuclease was achieved at low applied fields (25–100 V/m). Studies indicated that for maximum throughput, high field strengths and high flow rates are necessary. In addition, modifications to

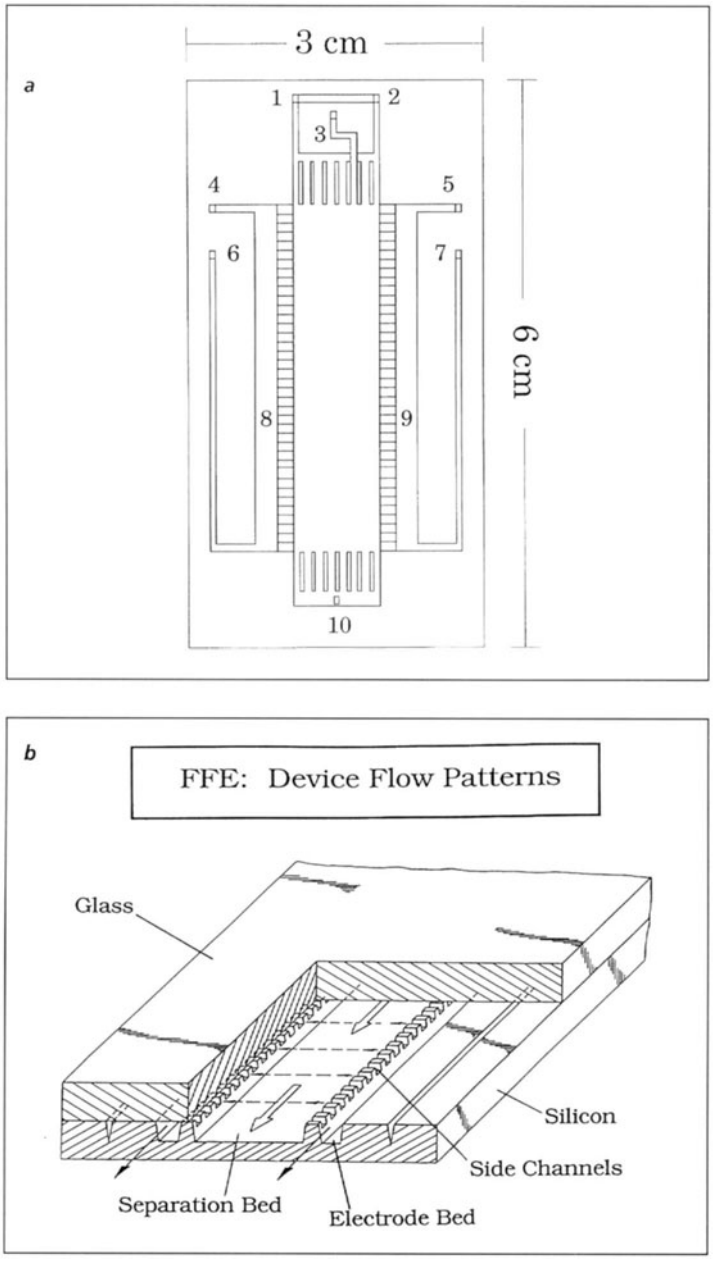

Figure 13 *(a) Schematic of a free-flow electrophoresis (FFE) microchip: 1,2 – carrier buffer inlets; 3 – sample inlet; 4,5 – side bed inlets; 6,7 – side bed outlets; 8,9 – side beds containing Pt electrodes; 10 – outlet. (b) Three-dimensional view of the inlet region on a FFE microchip*

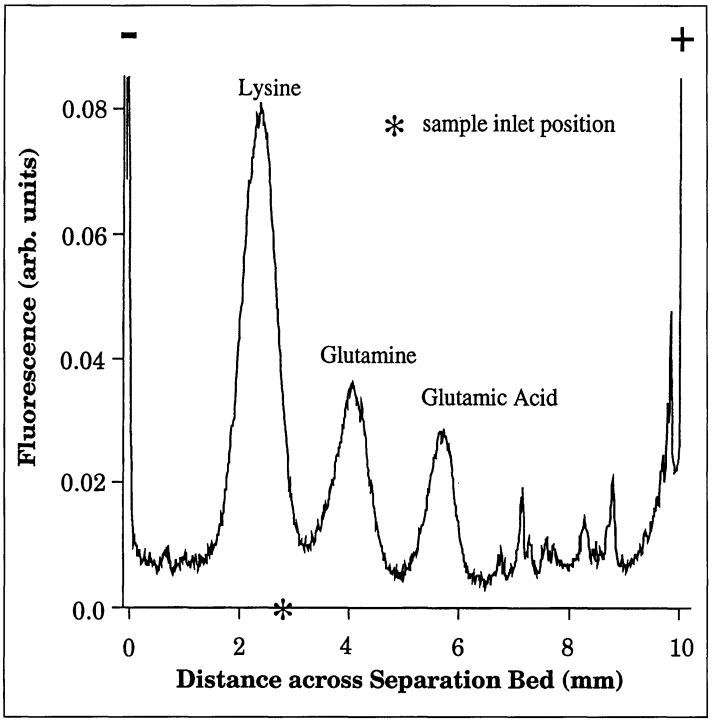

Figure 14 *Separation profile of rhodamine B isothiocyanate labelled amino acids (lysine, glutamine, glutamic acid), obtained 22 mm along the separation bed with an applied voltage of 50 V (current 7.5 mA). Sample and carrier flow rates were 0.2 and 5 μL/min respectively*

the silicon microstructure enabled efficient fraction collection subsequent to separation.

Much of the current interest in CE microchips has centred around their use in immunoassays. These assays are based on the separation of free and bound forms of antigen or antibody, and are commonly used for selective determination of molecular species at low concentration. The unique fluidic control system afforded by a chip format and reduced analysis times should allow for the development of fast, automated immunoassays for use "in-the-field". The first example of a microchip based immunoassay was presented by Koutny et al. in 1996 [80]. A competitive immunoassay for the analysis of cortisol in serum was performed in a fused-silica microchip. Fast, reproducible separations allowed the determination of cortisol in blood serum over the range of clinical interest. More recently, Harrison and co-workers have

demonstrated on-chip, homogeneous, immunological analyses for proteins such as immunoglobulin G (IgG) and drugs such as theophylline [81]. The problem of protein adsorption to channel walls was minimised through careful choice of buffer systems.

Chromatography

Chromatography encompasses a diverse group of techniques that permit the separation of closely related chemical components. The most fundamental classification of chromatographic methods is based upon the type of mobile phase used in separation. Consequently, two generic methodologies can be defined: *gas chromatography* (GC) and *liquid chromatography* (LC). In GC, elution is effected by the flow of an inert gaseous mobile phase. Many volatile, stable chemicals can be separated using this method. For example, GC is the most common technique used in the screening of drugs and narcotics, and it is estimated that over 200000 gas-chromatographs are in current use worldwide [82]. LC, on the other hand, utilises a liquid mobile phase. It has wider application than GC due to the fact that 85% of all known compounds are not sufficiently stable or volatile for use in GC. The modern forms of LC are generically termed HPLC (high-performance liquid chromatography). HPLC is unquestionably the most widely used separation and quantitative analysis technique, with annual sales of HPLC equipment of approximately 1 billion dollars [82]! HPLC techniques have undergone much development recently.

Both techniques appear to be particularly amenable to a micromachined format, and indeed, the very first example of a microfabricated chemical separation device was a gas chromatograph. As noted previously, this GC analyser was fabricated by Terry and co-workers at Stanford University in 1975 (over 15 years before the manufacture of the first microfabricated CE device) [48, 83]. Integrated onto a single silicon wafer (50 mm diameter) were a sample injection valve, a 150-cm-long spiral separation channel (30 µm deep and 200 µm wide), and a thermal conductivity detector (based on a nickel film resistor). Connections for a gas inlet and the detector were etched through the 200 µm silicon wafer. The GC chip was used in conjunction with the carrier gas supply and data processing apparatus, as shown in Figure 15. Simple separations of gaseous hydrocarbons were effected in less than 5 s.

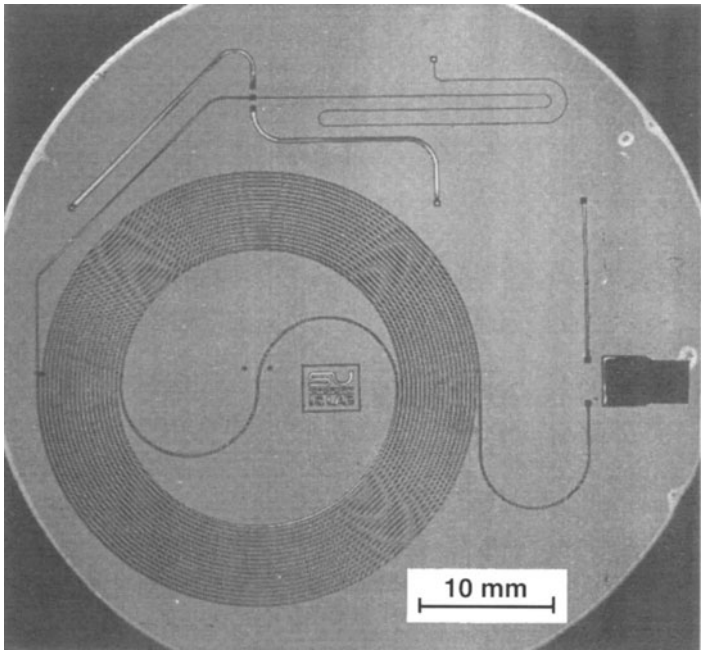

Figure 15 *Photograph of integrated Gas Chromatography silicon chip manufactured by Terry and co-workers [48]*

The microfabricated GC was small and compact, but performance and peak capacity were poor compared to conventional GC equipment used in the analysis of organic species. Consequently, the "analytical community" showed little interest in this new approach to high-speed, integrated chromatography [84]. The only other example of a microfabricated GC research in the literature is that of Reston and Kolesar [85–87]. Their approach utilises silicon micromachining and integrated circuit processing techniques. The GC system consists of a miniature sample injector, 90 cm rectangular column coated with a copper phthalocyanine (CuPc) stationary phase, and a dual detector (a CuPc coated chemiresistor and a thermal conductivity detector). Silicon micromachining is used to interface the injector and column. These miniaturised GC devices are able to separate NH_3 and NO_2 at ppm concentrations in less than 30 min [86]. More recently, Tjerkestra et al. have reported the fabrication of high performance GC columns [88]. These are made by isotropically etching half-circular channels in silicon substrates. These half-

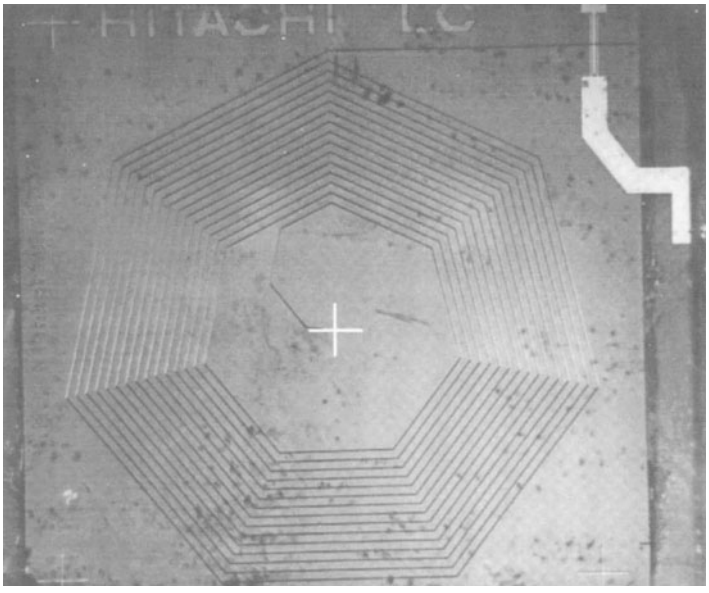

Figure 16 *Micrograph of Liquid Chromatograph microchip manufactured by Manz and co-workers [49]*

circular channels can be bonded on top of each other to yield channels with circular cross-section.

HPLC techniques have also undergone advances through the miniaturisation of component parts. For example, the use of packed and open microcolumns have increased separation efficiencies, but are difficult to implement, and generally result in long analysis times. Open-tubular column LC is an approach designed to reduce analysis times. According to theory, LC in open columns should allow for short analysis times with increased separation efficiencies compared to standard packed-column HPLC [89]. The first demonstration of a microfabricated, liquid-phase separation device was in 1990, by Manz and colleagues at Hitachi Ltd. [49]. They constructed a silicon LC microchip. Integrated on this chip was an open-tubular separation column and a conductometric detector. A micrograph of the LC microchip is shown in Figure 16. The open column has dimensions 6 μm × 2 μm × 15 cm, resulting in a total column volume of 1.5 nL, and the detection cell volume is 1.2 pL. Subsequent efforts at creating a workable HPLC chip demonstrated integration of a split injector, a packed small-bore column, a frit and an optical detec-

tor cell onto a silicon chip [90]. In this instance, the separation column volume was 500 nL, and the total dead volume less than 2.5 nL. Figure 17 illustrates a LC separation of fluorescein and acridine orange using this microchip. Cowen and Craston have also reported successful open-tubular LC separations on a silicon chip [91, 92].

At approximately the same time, Jacobson et al. reported open-channel electrochromatography on a glass microchip [93]. The internal surface of a microfabricated channel was chemically modified with octadecylsilane, producing an open-tubular reversed phase LC column. As for CE separations on microchips, EOF was used to load sample, and also to "pump" the mobile phase. Using fluorescence detection, a mixture of coumarin dyes was separated within 150 s at a linear flow rate of 650 µm/s. This study clearly demonstrated the potential of microchip devices in the separation of neutral molecules.

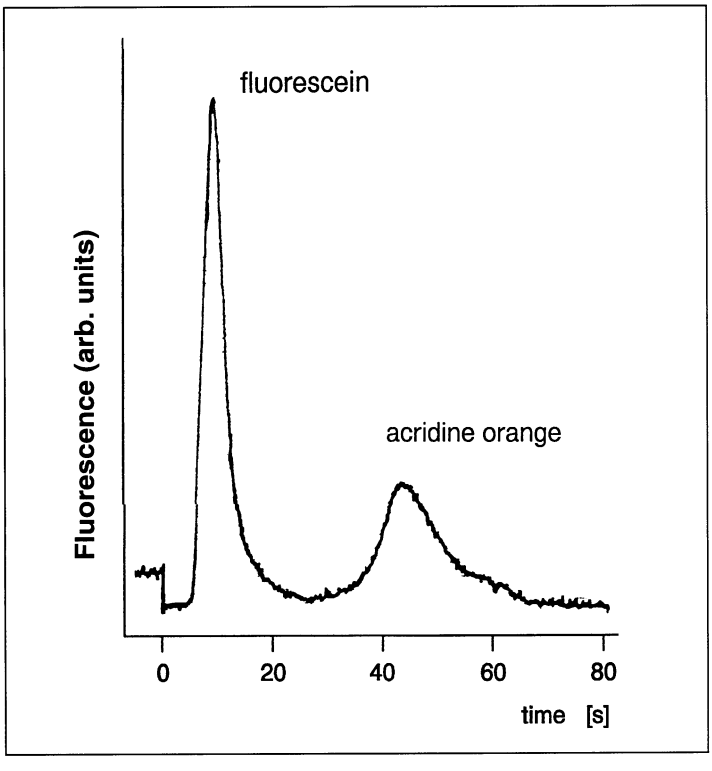

Figure 17 *LC separation of fluorescein and acridine orange using a packed separation channel. Sample: 100 µM fluorescein-Na, 100 µM acridine orange-HCl. Stationary phase: Nucleosil 100-5-C8. Injected volume before split: 60 nL.* F_{col}*: 3.2 µL* min^{-1}

Within the last 3 years micellar electrokinetic capillary chromatography (MECC) on planar, glass microchips has been reported by both von Heeren et al. [94] and Moore et al. [95]. In MECC, a surfactant above the critical micelle concentration is added to the running buffer. This allows the separation of uncharged solutes based upon differential partitioning [96]. The first demonstration of MECC on a microchip reported separations of three neutral coumarin dyes. Detection was performed using laser-induced fluorescence. At low applied electric fields highly reproducible separations were obtained. High electric field strengths, resulted in shorter analysis times but poorer separation efficiencies [95]. More recently, von Heeren and co-workers reported a comprehensive study of MECC on a glass microstructure. A cyclic channel geometry was used to effect the separation of six fluorescently labelled amino acids within a few seconds. More significantly, a chip-based MECC immunoassay for serum theophylline was presented. Compared to conventional MECC in fused silica capillaries, MECC microchip analyses were performed 1–2 orders of magnitude faster, with higher efficiencies [94]!

5.5 Detection

The scope of this chapter does not permit a comprehensive survey of all detection protocols used in microseparation science, and indeed detection is addressed in depth elsewhere in this volume. Nevertheless, the chapter would be incomplete without cursory reference to some of the detection modes used by researchers in the field of μ-TAS technology.

The adaptation of a number of detection protocols to measurement in small volumes (and therefore small numbers of species) has accompanied the development of μ-TAS. Detection volumes may be as low as a few hundred attoliters in some microfabricated systems, and the very nature of microseparations means that detection times must also be rapid.

Small volume optical detection is generally based around either absorption or fluorescence measurements. Absorbance is directly proportional to the optical pathlength as well as the sample concentration. Consequently, the major difficulty faced in small volume absorption measurements is the ability to probe small volume cells whilst maintaining a long enough pathlength.

With microfabricated CE chips, this pathlength problem is enhanced (due to reduced channel dimensions) and, to date, fluorescence detection has been the principal optical detection method (in more than 95% of all studies). Although fluorescence is inherently a far more sensitive technique [12], absorbance detection has a much wider applicability (i.e. not all species that absorb radiation fluoresce), and is therefore of importance as a detection protocol. In addition both absorption and fluorescence detection are generally highly specific, and less prone to interferences than their electrochemical counterparts.

Alternative approaches have been developed to enable on-chip absorption measurements. Verpoorte and Manz reported a micromachined absorbance Z-cell for use in liquid chromatography [97]. In this design, the crystal planes of silicon (111) are used to form "micro-mirrors" which effectively extend the optical pathlength. CE microchips, on the other hand, cannot be fabricated in silicon due to adverse electrical characteristics. Hence, an alternative strategy to launch and collect light is required. Liang and co-workers have recently demonstrated the use of a planar, optical U-cell in glass, for both absorption and fluorescence detection [98]. The cell provides a ten-fold improvement in absorbance detection limits by probing the channel in a longitudinal rather than transverse direction. The cell also allows for improvements in the S/N ratio of fluorescence measurements.

Other approaches to integrated optical detection have recently been reported. For example, Hoppe and co-workers have described a novel method of fabricating integrated optical waveguides for use in fluidic microsystem [99]. In addition, Bruno et al. have reported the construction of μ-optics specifically geared for use in μ-TAS. These components include light emitting diodes, gradient index optics and thermo-optical devices [100]. Weigl and co-workers have also developed a novel optical chemical detection protocol for use in silicon flow structures. This device allows for the continuous monitoring of analytes within particle laden solutions, using either fluorescence or absorption spectroscopies [101].

Other generic detection methods have been used in conjunction with μ-TAS. The original gas chromatograph developed by Terry and co-workers incorporated a microfabricated thermal conductivity detector [48, 83]. Furthermore, among the earliest detector cells for liquid-based analysis were devices that combined solid state chemical sensors with small-volume sample

chambers defined in silicon [102]. Interestingly, Burggraf has also demonstrated refractive index detection for CE on glass microchips [103]. Although detection limits are poor compared to more conventional protocols, refractive index measurements afford the possibility of truly *universal detection*.

As stated, the primary consideration when choosing a detector for a μ-TAS is that it can probe small volumes. Electrochemical detection protocols have been used within a μ-TAS format for this very reason [104, 105]. Furthermore, the whole electrochemical sensor itself can be made very small. This can be of importance if portability is a system requirement.

A key aspect of chemical analysis is *structure elucidation*. Mass spectrometry (MS) is one of the most effective tools available for this purpose. Very recently electrophoresis microchips have been successfully combined with the technique of electrospray mass spectrometry (ESI-MS) [106, 107]. Karger and co-workers demonstrated high sensitivity (low nanomolar) microchip ESI-MS in the analysis of proteins, and soon after, Ramsey reported a method of generating electrospray from solutions emerging from microchannels etched on planar glass substrates. This approach to detection extends the applicability of μ-TAS to molecules which are non-fluorescent, and leads to the possibility of high-throughput MS analysis in screening and diagnostic applications.

5.6 The "laboratory-on-a-chip" – fact or fiction?

The ultimate μ-TAS will combine all aspects of the analytical process. Component processes such as sampling, sample pre-treatment, sample transport, chemical reaction, product separation, product detection and product isolation will all be performed in an automated manner on a single microchip device. The previous section has summarised the dramatic advances made in "microseparation" technology over the past 7 years. Classical separation methodologies (CE, LC and GC) can now be effectively converted to a microchip format, leading to improvements in analysis time and separation efficiency (a direct result of the miniaturisation process). In addition, other potential μ-TAS components such as reactors, micropumps, sieves, heaters, filters, microvalves and detector cells have all been fabricated and characterised on a micron scale. Consequently, the next step is the integration of these

modules to create a complete, integrated analytical system (ideally on a microchip).

To date there have been few real attempts to fabricate a fully integrated μ-TAS (based on a "*react-separate-detect*" principle). As recognised in the previous section, Ramsey and co-workers reported both pre- and post-separation reactions on glass microchips [64, 65]. For the pre-separation reaction, a fast derivatisation was performed by mixing reagents in a dilated section of channel (1 nL volume). This reaction, although extremely simple (i.e. diffusional mixing two reagents at ambient temperature), established the feasibility of integrated reactions and separations on a single microchip. As Ramsey says, the system accomplishes the same things that a laboratory technician or an automated robotic system could do. Specifically, the microchip mixes two reagents, incubates them for a given time, injects the product mixture into an analytical system and presents the results to a digitising detector. All these processes are performed at an extremely high level of precision.

Many biological and clinical problems will benefit from the ability to perform rapid, automated analyses on minute quantities of material in a high-throughput fashion. As noted, fulfilment of the Human Genome Project will require an increase in processing speed of up to 500-fold by the year 2000 [9]. Consequently, miniaturised, integrated DNA analysis systems should provide a route to faster, cheaper analyses. Demonstration of an integrated device of this kind was first provided by Jacobson et al. in 1996 [108]. An integrated monolithic device that performs restriction digestion followed by an electrophoretic sizing of the products was fabricated in glass using standard photolithographic methods. The DNA plasmid *pBR322* was mixed with the *Hin*fI restriction enzyme in a 700 pL reaction chamber. Subsequent to digestion, the DNA fragments are injected into a 67 mm separation channel and sized. Digestion of the pBR322 plasmid by *Hin*fI, and fragment analysis were completed within 300 s. The success of this chip based DNA analysis indicated the possibility of miniaturising more sophisticated biochemical procedures.

The polymerase chain reaction (PCR) has revolutionised the biological and medical sciences since it was formally introduced at the Cold Spring Harbor 51st Symposium on Quantitative Biology [109]. The PCR process allows virtually any nucleic acid sequence to be generated *in vitro* in ab-

undance. This is done by repeated cycles of heating and cooling of sample material and enzyme in a reaction chamber. Although PCR is simpler, faster and more flexible than traditional cloning techniques, it is limited by instrumental technology. Conventional thermal cycling systems require long cycling times due to their large thermal masses (approximately 2–3 h for a 30 cycle amplification). In addition, the physical size of thermal cyclers has forced PCR to remain a laboratory technique. A number of microfabricated PCR devices have been proposed in the last 4 years. PCR amplification has been performed in 4–12 µL silicon/glass reaction chambers placed in larger thermal cyclers [110], in 20–50 µL microfabricated silicon chambers with integrated heaters and temperature control [111], and on a 25 µL drop of solution above a microfabricated heater [112]. All these devices call for fast, portable PCR.

The integration of miniaturised PCR amplification and ultrafast DNA analysis (two fundamentally different processes) on a single microchip device would allow for vastly reduced analysis times, instrumental portability, reduced sample requirements and high-throughput. All these characteristics are critical for the completion of the Human Genome Project. In addition, a device of this kind could genuinely be termed a *laboratory-on-a-chip.*

A first generation device of this kind has recently been reported by Woolley et al. [113]. An integrated PCR-CE microdevice was fabricated and shown to perform ultra-fast amplification followed by electrophoretic DNA analysis, with a complete absence of manual sample transfer. Figure 18 shows a schematic of the integrated PCR-CE microdevice. The CE manifold is a glass microchip containing microfabricated electrophoresis channels (separation channel – 8 µm × 100 µm × 46 mm filled with a HEC sieving matrix). The microfabricated PCR reactor has an etched silicon reaction chamber, LPCVD doped polysilicon heaters, and gold/titanium heater contacts. Disposable polypropylene liners were inserted into the reactor to minimise contamination and adsorption effects. The PCR chamber and CE manifold were coupled at one of the sample reservoirs. Electrophoretic injection directly from the PCR chamber through the injection channel was used as an "electrophoretic valve". The functionality of the system was initially demonstrated by amplifying a 268 bp β-globin target cloned in M13. A 30 cycle PCR amplification followed by electrophoretic separation, and fluorescence detection took less than 20 min! In addition, a rapid assay for genomic *Salmonella* DNA was

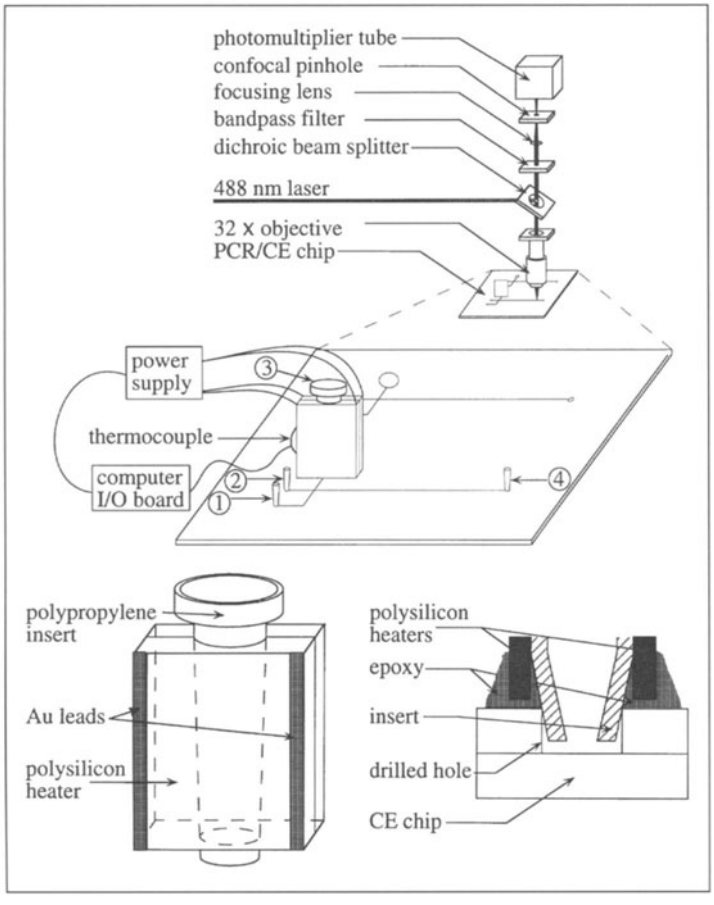

Figure 18 *Schematic of an integrated PCR-CE microdevice. (A) Laser-excited confocal fluorescence detection apparatus and an integrated PCR-CE microdevice. (B) Expanded view of the microfabricated PCR chamber. (C) Expanded cross-sectional view of the junction between the PCR and CE devices*

performed in under 45 min (Fig. 19). Finally, the authors established the feasibility of real-time monitoring of PCR target amplification. The demonstration of a functional integrated PCR-CE microdevice is a significant step toward the complete integration of DNA analysis, and more generally chemical analysis.

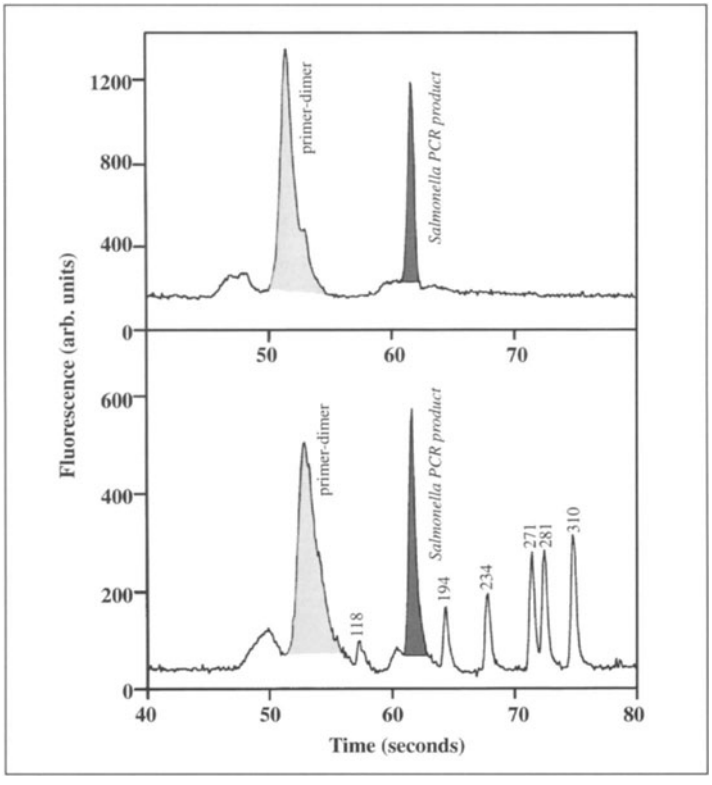

Figure 19 *High-speed integrated PCR-CE microdevice assay of genomic Salmonella DNA. (Top) Chip CE separation of the Salmonella PCR product was performed immediately following a 39-min PCR amplification in the integrated PCR-CE microdevice. The primer-dimer peak (light grey) appears at 51 s and the PCR product peak (dark grey) appears at 61 s. Total analysis time for the Salmonella sample using the integrated PCR-CE microdevice was under 45 min. (Bottom) Sizing of the Salmonella PCR product (1 : 100 dilution) using ϕX174 HaeIII DNA (1 ng/μL) in a separate CE chip*

5.7 Conclusions

The title of a review article over 6 years ago posed the following question: "Miniaturisation of Chemical Analysis Systems – A look into Next Century's Technology or Just a Fashionable Craze"? [1]. At the time, the answer was not an obvious one. Fundamental diffusional and hydrodynamic theories suggested that *faster* and *more efficient* separations would result, by miniaturising

both chromatographic and electrophoretic processes [114]. In addition, miniaturisation of the analytical process reduced reagent requirements and afforded the possibility of fully integrated and automated systems. However, little experimental work had been undertaken to address these theoretical claims, and thus the authors were incapable of answering their own question. Six years on, and we can now begin to answer that question. Miniaturisation is surely not a fashionable craze? Or if it is, it is one which is standing the test of time! However, it is still difficult to foresee the precise impact that μ-TAS will have on the analytical sciences of the 21st century.

The ultimate challenges are clear: Can a complete analytical procedure be miniaturised? In addition, can the component processes be integrated on a single device (most likely a microchip)? And finally, can the operation of the complete process be automated? The research summarised in the preceding sections demonstrate technological advances in all of these three areas.

The development of microseparation technology has been the area of most obvious development over the past 6 years. The separation of both charged and neutral species can be now effected using chip-based formats of many techniques. These include CE, SCCE, FFE, micellar electrokinetic chromatography, open-channel electrochromatography and packed bed, liquid chromatography. Through direct experiment, the theoretical predictions about separation speed and efficiency have now been validated, and the use of μ-TAS as a highly efficient alternative to their conventional counterparts can be justified. In addition, the gains in separation speed and efficiency now permit CE microchips to be used in similar ways to chemical sensors for certain applications [61].

Integration of component processes will be key to the continued development of μ-TAS. In many ways the "separation" element of the analytical process is now well defined. For example, the construction and operation of a CE microchip is now a simple task for many research groups. The real challenge is to link in the sample pre-treatment, detection and data processing to the separation stage. Of these, sample pre-treatment appears to hold the most scope for development. Although sample manipulation through electrokinetic pumping mechanisms is well understood, the analysis of real samples will require sophisticated handling steps, such as filtration [44], mixing [47], heating [113]. Many of these components have been reported

individually, but very few studies have approached analysis in a truly integrated manner.

Fortunately, more contemporary research is now beginning to address the issue of integration. As shown previously, microchip devices for immunoassays [81] and DNA restriction fragment analysis [108] have been reported. However, the recent demonstration of an integrated PCR-CE microchip is probably the most significant step towards the true *laboratory-on-a-chip* [113]. This integrated device performs a diagnostically relevant and sophisticated reaction followed by the separation and detection of reaction products, with the absence of manual transfer. The only point of human contact is the loading of the reactants into a reaction chamber at the very start. This is significant since the whole analytical process then becomes automated. The final steps in creating a fully integrated/automated device will be the integration of all sample handling and product detection on a single microdevice. This may not be too long away.

The generality of the miniaturised analysis system concept makes it applicable to many areas of science and technology. These include industrial process control, environmental analysis, medical and clinical diagnostics and forensics. At the start of the decade the clinical diagnostics market alone was over $9 billion US dollars annually, and the market for chemical sensors was estimated at around $5 billion US dollars [2]. These figures give an idea of the size of the potential markets that μ-TAS can tap into. Indeed considerable corporate research is currently focused on the development of μ-TAS technology. Large scale business activities include those of Caliper Technologies, Affymetrix, Ciba-Geigy, Soane Biosystems, Nanogen, Hitachi, Orchid, Shimadzu Scientific Instruments and PerSeptive Biosystems.

In conclusion, it is fair to say that the transition between the conventional, "bench-top" approach to chemical analysis and the μ-TAS described in this chapter will undoubtedly prove to be as significant an advancement as the microelectronic revolution was over three decades ago. However, to finish on a more futuristic note, imagine a hapless analytical chemist who was lost at sea in the year 1990 *anno domini*. After many adventures and trials he at last finds his way back to his native laboratory, 20 years later. The job of today's μ-TAS researcher will be to make him feel like Gulliver landing on the Island of Lilliput and exclaiming "what on earth are all these tiny, strange objects!"

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