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REVIEW

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Miniaturized total analysis systems for biological analysis

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Abstract This review article discusses and documents the basic philosophies, concepts and current advances in the field of μ-TAS development, with special emphasis on applications in the arena of biosciences. After a brief overview of miniaturization theory and fabrication techniques, areas of microfluidic component development, detection protocols, biochemical assays, and integrated biological analyses are addressed.

1 Introduction

The precise and accurate determination of chemical or biological parameters has always been of central importance in science. However, over the past twenty years a need for rapid, on-line measurements at low concentrations has developed within the fields of chemical production, DNA analysis, drug discovery, pharmaceutical screening, medical diagnostics and environmental analysis [1–5].

In the real world, an analyte of interest (whether is be a small organic molecule or a much larger biopolymer) is usually present as a minor component in a complex mixture. This means that discrimination of the analyte from potential interferences is usually the critical step in the complete analysis process. Chemical sensors provide a direct solution to this need [6]. Ideally, a chemical sensor transduces molecular information relating to a particular species into electronic information. Furthermore it performs this task in real time and at the exclusion of all other components in the sample matrix. An alternative approach to molecular identification is to incorporate a separation step prior to transduction. This lessens selectivity requirements in detection and improves sensitivity (due to reduced background levels). This strategy has been developed into the concept of a Total (chemical) Analysis System (TAS) [1]. Ideally, a TAS performs all the component stages of a complete analysis in an integrated and automated fashion. These stages can include sampling, sample pre-treatment, chemical reactions, analytical separations, analyte detection, product isolation and data analysis. The philosophy of TAS has enabled enhancements in on-line chemical analyses, however, significant drawbacks still exist. These include slow sample transport, high reagent consumption, poor separation efficiencies and the need to fabricate interfaces between distinct components.

Improvements in overall analytical performance can be achieved by minimizing the scale on which the analysis is performed. The concept of a 'downsized' TAS was first suggested by Manz and Widmer in 1990, and christened a μ-TAS (Miniaturized Total (chemical) Analysis System) [7, 8]. In essence, a μ-TAS is a device that improves the performance of an analysis by virtue of its reduced size. Typically, a μ-TAS is a microchip or micro-device fabricated using conventional micromachining technologies. Enhancements in performance relative to TAS can be shown through experiment and theory [7, 8]. For example, miniaturization of flow manifolds leads to reduced reagent consumption, greater separation efficiencies and reduced analysis times. Although by definition μ-TAS are physically small with respect to their conventional analogues, this is generally not the driving force for development. The notions of improved analytical performance, component integration, increased throughput and automation are far more important. Clearly, the size is important in niche areas, such as 'point-of-care' diagnostics and 'in-the-field' analysis, but even then performance gains will determine their applicability.

During the past decade, the concept of a μ-TAS has been developed, refined and applied to a variety of chemical and biological problems. Many of these have been detailed in some excellent, recent reviews. These include reports of integrated chip-based separation methods [9–11], biochemical applications of microsystems [1] and the clinical potential of chip-based analysis systems [12]. In addition, more complete and up-to-date coverage of the area of μ-TAS can be found in the proceedings of the bi-

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ennial International Symposium on μ-TAS (μ-TAS 2000 will be held in May 2000 at the University of Twente, Netherlands) [13].

2 Miniaturization vs. performance

2.1 Separations

As previously stated, the primary gain of miniaturization is enhanced analytical performance. In chromatography and electrophoresis analytical performance is normally judged through the analysis time *t*, and the separation efficiency (expressed as the number of theoretical plates *N*). Using capillary electrophoresis (CE) as an example the analysis time is given by

$$t = \frac{L^2}{\mu V_s} \tag{1}$$

where L defines the distance between the point of injection and point of detection, μ the vector sum of the electroosmotic and electrophoretic mobilities of a given species, and V_s the potential drop across the separation channel. Inspection of Eq. (1) clearly demonstrates that a reduction in L will significantly reduce the total analysis time.

The number of theoretical plates describes the ability of a separation system to discriminate between different species. Longitudinal diffusion (caused by intra-channel concentration gradients) and extra-channel band broadening due to sample injection and detection all act in real systems to reduce this ability [14]. Consequently, *N* can be defined according to

$$N = \frac{L^2}{\sigma^2} \tag{2}$$

where σ^2 is the sum of the variance contributions of longitudinal diffusion, injection and detection. All three contributions to band broadening are unavoidable in a real system. However, injection and detection variances can usually be made much smaller than a quarter of the diffusional variance. Consequently, in a simple treatment it is valid to consider σ^2 as being solely due to diffusional processes. In this case, with an analysis time *t*, the band variance at the detector is given by

$$\sigma^2 = \frac{2DL}{v} \tag{3}$$

where D (m^2/s) is the diffusion coefficient of the molecular species and ν is its total migration velocity (m/s). Substitution of Eq. (3) into (2) yields a more complete expression for *N*, i.e.,

$$N = \frac{Lv}{2D} = \frac{L\mu E}{2D} = \frac{\mu V_s}{2D} \tag{4}$$

This expression is significant since it states that although the separation efficiency increases as a function of applied voltage, there is no dependency on the channel dimensions. In other words, miniaturization should *not* directly influence separation efficiencies.

A closer analysis of this situation demonstrates that this is not the whole truth. Although Eq. (4) shows that *N* can be improved by increasing the potential drop across the separation channel, this cannot occur indefinitely. In practical applications Joule heating effects impose a maximum limit on V_s. At high applied potentials radial temperature gradients are produced within the channels. These act to increase radial diffusion and thus increase σ^2 [15, 16]. The heat *Q* (W/m) generated per unit length in a rectangular channel of height *h* and aspect ratio *a* is given by

$$Q = \frac{V_s^2 h^2 a}{L^2} \lambda c \tag{5}$$

where λ ($m^2/mol\Omega$) is the molar conductivity of the buffer and *c* (mol/m^3) its concentration. This expression shows that *Q* increases as a function of V_s, as expected [10]. However, a reduction in both *h* and *a* (especially *h*) will reduce *Q* and thus allow higher field strengths to be used (which will afford an increase in *N* if desired). In fundamental terms, since heat is generated uniformly within the fluid volume element, but can only be dissipated across the immediate boundaries of the electrophoresis channel increasing the surface-to-volume ratio provides the dominant route to improved heat dissipation. Eijkel et al. [10] have extended the treatment above and determined the maximum rate of plate generation as

$$\left(\frac{N}{t}\right)_{\max} = \frac{1}{h^2} \frac{\mu^2}{\pi \lambda c D} \lambda c \tag{6}$$

This reinforces the idea that the maximum rate of plate generation can be dramatically increased by decreasing channel heights. Interestingly, as Eijkel notes, the rate of plate generation is independent of channel width, implying that wide channels can be used without any loss in performance.

In conclusion, this simple treatment shows that analytical performance in CE systems can be improved in three primary areas:

1. Analysis times can be reduced through a reduction in channel length or an increase in separation voltage.
2. Plate numbers can be increased through an increase in separation voltage (as required in 1).
3. Heat generation can be reduced by reducing channel heights.

2.2 Reactions

Generally, the key components of a complete chemical analysis are a chemical or biological reaction, and a pre- or post-reaction separation. It has been shown that miniaturization of instrumental dimensions dramatically improves separation efficiencies and analysis times, but how do chemical reactions behave on a small scale? This question is not easy to answer due to huge diversity in the needs and nature of chemical and biological systems. Consequently, only some generic features will be addressed.

For homogeneous reactions mixing together two components could be considered the fundamental process. On the macroscale, mixing generally involves two steps. First, segregation creates a heterogeneous mixture with a finely dispersed structure. Second, diffusion (random molecular motion along a concentration gradient) between adjacent domains leads to a mixture which is homogeneous at the molecular level [17]. To ensure that the entire process is sufficiently fast, the size of the pure fluid elements has to be reduced to a level where the rate of diffusion is significant. In the macroscopic mixing of low viscosity fluids (high Reynolds number regimes) the first phase is achieved through the creation of turbulence. Mixing similar fluids in micron-sized vessels normally falls into the laminar flow regime (low Reynolds numbers) where viscosity dominates over inertia and dampens out irregularities in flow patterns. This means turbulence is unattainable and mixing can only occur via molecular diffusion.

The diffusion of any molecular species is characterized by its discrete diffusion coefficient, D (m^2/s). In the simple case of a spherical molecule,

$$D = \frac{kT}{6\pi\eta r} \tag{7}$$

where k is the Boltzmann constant (1.38×10^{-23} J/K), T the absolute temperature (K), η the absolute solute viscosity (kg/m · s) and r the hydrodynamic radius. A small molecule, with a molecular weight between 500 and 1000 would be expected to have a diffusion coefficient of approximately 5×10^{-10} m^2/s. Under these conditions (without turbulence), the molecule will diffuse across a dimension of 1 mm in approximately 30 min (2000 s). If the dimension is reduced to 50 μm the diffusion time drops to about 5 s. And if the dimension is further reduced to 1 μm the diffusion time will become approximately 2 ms.

This brief analysis illustrates that purely diffusive mixing of reagents is only viable for reactor dimensions well below 100 μm. However, at these small dimensions mixing (although solely diffusion mediated) is extremely fast, and should allow for reaction times to be determined by inherent reaction kinetics rather than the time it takes for reactant species to meet in solution.

In applications where heating and cooling is essential, miniaturization is also advantageous. According to Fick's law, the time needed for heat dissipation is directly proportional to the second power of the channel (or reactor) depth for a flat rectangular channel. Although this condition assumes an infinite heat capacity reservoir surrounding the fluid element, it demonstrates that heating and cooling can be performed on ultra-short timescales within microfabricated environments.

2.3 Scale of integration

It is of interest to consider potential gains in the scale of integration (device density) that miniaturization presents. This has obvious implications in device cost, high-throughput applications and parallel processing. Device density scales as a function of $1/d^2$, where d^2 defines the device footprint. Thus, if d is 1 cm, 1 device per cm^2 is possible. This increases to a theoretical 100 devices per cm^2 for a dimension of 1 mm, and 10^8 devices per cm^2 for a dimension of 1 μm. Clearly, smaller dimensions are desirable, but can only be attained with superior fabrication technologies.

3 Microchip fabrication and materials

Components (e.g. channels and reaction vessels) of a μ-TAS can often be created with the diverse planar fabrication processes originally developed in the microelectronics industry [18]. Since most features are relatively large (> 1 μm), fabrication is relatively straightforward and can be achieved with standard methodologies. Descriptions of basic and sophisticated fabrication protocols have been detailed [19, 20].

In the simplest (and most common) case, a channel pattern is transferred to a planar substrate using a combination of photolithography, wet-etching and bonding. The basic procedure starts with the deposition of a durable, photosensitive polymer (positive-photoresist) onto the substrate surface. The coated surface is then exposed to UV radiation through a mask. Subsequent development in an organic solvent allows the removal of exposed portions of photoresist leaving a polymerized resist pattern with high chemical resistance. This resist mask is used to define one layer of the desired microstructure. Etching allows the two-dimensional photoresist pattern to be transferred to the substrate material. 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). The conventional isotropic process generally yields sloping walls and relatively low aspect ratio features. Anisotropic etching processes can be used to provide vertical walls with much higher aspect ratios [21, 22]. Aspect ratios (ratio of height to width of the channel) can vary greatly from shallow, wide channels to narrow, deep channels [23, 24].

The final stage in the fabrication of a basic μ-TAS is bonding: the assembly of the substrate materials (e.g. silicon-to-silicon, glass-to-silicon, silicon-to-oxide and glass-to-glass) to form the enclosed chip structure. Anodic bonding is commonly used to bond glass and silicon substrates. This procedure utilizes 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 substrates. Furthermore, the planarity of the substrate surfaces generally means that bonding is a simple and efficient final step in the manufacture of μ-TAS. Conventional thermal bonding techniques are often time-consuming and require high temperatures that can interfere with immobilized chemicals and features on the chip. Recent

innovations have addressed this problem by moving to lower temperature regimes. These approaches have included the use of an adhesive sodium silicate layer (cured for 1 h at 90 °C or overnight at room temperature) [25], and methods involving 1% HF and pressure [26]. Both yield similar results to those achieved using high temperature bonding protocols.

Bonding is a crucial step in μ-TAS fabrication. This is particularly relevant when biological components (e.g. proteins, enzymes and oligonucleotides) need to be immobilized onto the surfaces of micro-features. Conventional techniques for the thermal bonding of glasses usually operate at temperatures well above those at which most biological components are stable. Although bonding methodologies at temperatures below 100 °C have been successful, these are often not universally applicable, or require the use of reagents that are not biocompatible [25]. An obvious solution to this problem is to use polymers to form part or all of the microchip.

Polydimethylsiloxane (PDMS) has shown great promise in such applications. This elastomeric polymer produced by Dow Corning has several attractive features [20]: it can be easily molded, it is optically transparent (well into the UV), it is durable, chemically inert, cheap, non-toxic and chemically stable between –55 and 200 °C [20, 27, 28]. Creation of microstructures with this polymer is easily accomplished. A 10 : 1 mixture of the base and hardener is deaerated under vacuum, poured over a negative image (i.e. a replica mold) of the desired structure and allowed to cure. Curing can take place at 90 °C for one hour or overnight at room temperature. The polymer is removed from the mask, which can be reused. Enclosed channels are formed when PDMS forms a seal with another substrate such as glass. An advantage of PDMS is that the seal between upper and lower substrates does not need to be permanent. This means that the PDMS layer can be lifted to allow blockages to be removed (impossible on a sealed chip). If the application requires a more permanent seal, treatment of bonding surfaces with an oxygen plasma allows PDMS to be irreversibly sealed with a variety of substrates including PDMS, glass, silicon, quartz, polyethylene and glassy carbon [29]. Chips made with PDMS are easier and cheaper to produce than those made exclusively with glass [29]. Whitesides and co-workers have demonstrated electroosmotic flow (EOF) in oxidized PDMS channels [29]. This polymer has also been used as a negative resist in order to cast further structures [30, 31]. Although PDMS can withstand high temperatures, it is unable to dissipate heat as readily as glass.

Another polymer of interest is SU-8, an epoxy resin produced by Shell [32, 33]. Once cured, the polymer can be exposed to UV radiation, developed and hard baked to render it inert to most solvents and metal etches. The use of SU-8 allows high aspect ratios to be created without resorting to exotic fabrication techniques [32].

Polymethylmethacrylate (PMMA) has also been used to form microfabricated structures [31, 34, 35]. Whereas PDMS cures to give an elastic polymer, PMMA forms a more rigid construct [31]. PMMA can be poured over a negative mask or exposed soft X-rays to form the desired microstructures, (again with high aspect ratios) [31, 34]. Ford et al. [34] have used PMMA to create microstructures for electrophoretic applications.

Pouring molds (replica molding) can be classed in the larger category of soft lithography [36–39]. Soft lithography encompasses all techniques used to form microstructures without etching silicon based substrates. Advantages of these methods include ease of fabrication, low cost, and multiple use of molds. An additional benefit of soft lithography is that it is not limited to planar surfaces. For example, Brittain et al. have used soft lithography to pattern optical fibers [40]. Whitesides and co-workers have written several recent reviews on soft lithography (covering microcontact printing, micromolding in capillaries, microtransfer molding, solvent assisted micromolding, replica molding, and near-field contact-mode lithography) [39–41]. A few highlights of these techniques will be given.

One soft lithography method of great interest is microcontact printing (μCP) [38]. μCP uses a negative PDMS mold, or stamp, of the desired pattern. The stamp is dipped into an ethanolic solution containing alkylthiols and placed on a substrate covered in a thin layer of gold. The alkylthiols form a self-assembled monolayer (SAM) where they contact the gold surface. These SAMs protect the gold surface while the chip is etched using ferri/ferrocyanide etchant. μCP has been successfully used to create very small features such as electrode arrays [38].

Hot embossing is a similar soft-lithographic technique [37]. Molds are stamped into semi-finished polymers under vacuum and at temperatures above the polymer's glass temperature. Examples of structures made using μCP and hot embossing are shown in Fig. 1.

New fabrication techniques using stacked functional laminates have been presented for more structurally complex systems [42]. Individually fabricated components (e.g. pumps, valves and reactors) can be assembled in any order to form a microfluidic device. The laminate layers can be fabricated using a variety of techniques, such as injection molding. This format has been used to make a chromium metal sensor and a dialysis membrane [42]. Devices have channel widths ranging between 100 and 500 μm and channel depths between 125 and 250 μm.

An extremely interesting facile technique that allows patterning inside channels has recently been presented by Whitesides [43]. Under conditions of laminar flow, reactions can take place at the interface between two or more liquids flowing side by side down a channel. Etching, electrode formation, and reactions have all been accomplished within pre-existing microfabricated channels. Figure 2 illustrates the in situ fabrication of gold electrodes in an isotropically etched microchannel.

LIGA (*LI*thographie, *G*alvanoformung, und *A*bformung) describes the major steps in microlithography: lithography, electroforming, and molding [35, 36]. In this procedure a pattern is transferred to radiation sensitive polymers. X-rays are the preferred radiation source as high resolution is obtained. After exposing and develop-

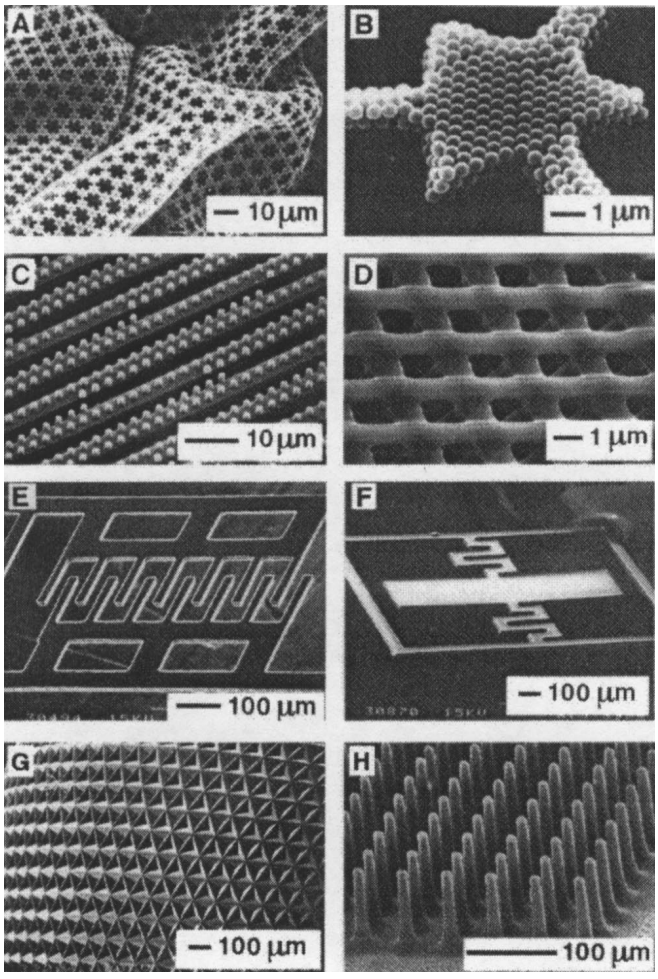

Fig. 1 Several examples of micromolding (A, B – MIMIC; C–F – μTM; G, H – replica molding). **A,** polyurethane structure fabricated using MIMIC on SiO_2 surface. **B,** MIMIC fabricated microstructures containing crystallized polystyrene beads. **C/D,** two and three layer microstructures fabricated by TM. **E/F,** SEM images of structures fabricated by μTM. **G,** hemispherical structure formed by replica molding against PDMS. **H,** high aspect ratio microstructure fabricated by replica molding (Reprinted with permission from [20])

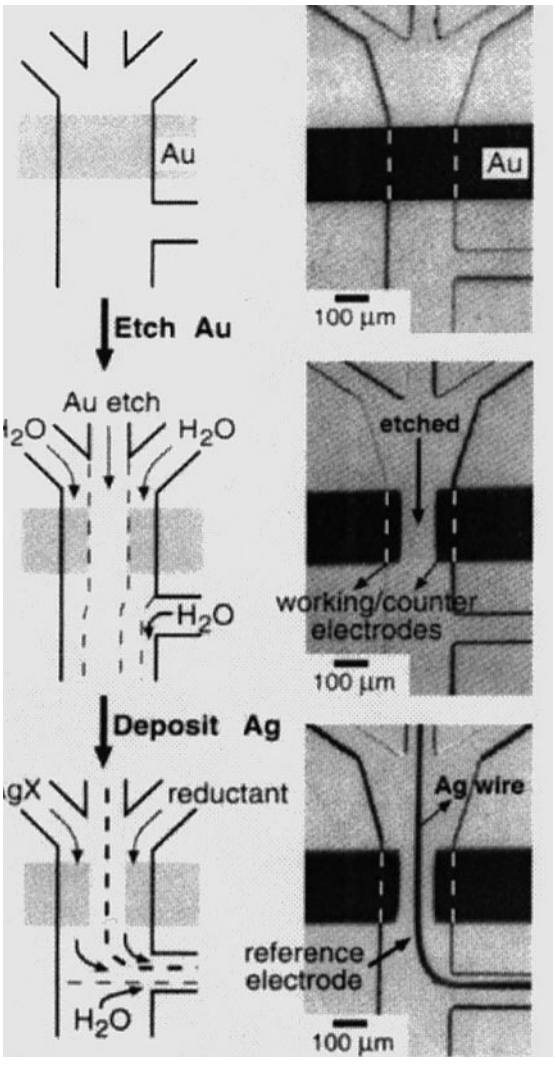

Fig. 2 The formation of a three-electrode system within a channel as shown by optical micrographs. Using a three-phase laminar flow system two gold electrodes are formed by etching a previously deposited gold strip and using a two-phase laminar flow system a silver reference electrode is fabricated (Reprinted with permission from Kenis PJA, Ismagilov RF, Whitesides GM (1999) Science 285:83–85. Copyright 1999 American Association for the Advancement of Science) [43]

ing the pattern, a metal mold is fabricated using electroplating. This results in an extremely accurate copy of the pattern. The metal mold can be used in mass production applications such as hot embossing or injection molding [35, 37, 44]. This approach has been used to create microfabricated capillary electrophoresis devices, for example using PMMA as the X-ray resist [34].

Other polymeric materials used in microsystem applications include conducting, or conjugated, polymers, such as polypyrrole [45, 46]. These polymers are also electroluminescent and can be cast in thin films on planar surfaces so their use as an integrated light source is an exciting (miniaturized) alternative to bulky lasers or lamps [46]. This class of polymer could also be used to fabricate transistors, diodes, chemical sensors and batteries [46].

4 System components

4.1 Channels

The fundamental component of a microfluidic chip is the channel network through which all samples flow. Channels can be made in a variety of substrates ranging from silicon to numerous types of polymers, such as PDMS. Fabrication was discussed more fully in Section 3. Channels are normally trapezoidal or rectangular in cross section, with dimensions ranging from hundreds of nanometers to hundreds of microns.

4.2 Detectors

The adaptation of conventional detection protocols for measurements in small volumes has closely accompanied the development of μ-TAS. Indeed, it has long been realized that ultimate size limits for μ-TAS are primarily set by the system detector. For example, when performing CE on a typical microfabricated device, injection volumes typically range between 10^{-14}–10^{-10} dm^3. At a diagnostically relevant target concentration of 10 nM, this results in between 100 and 10^5 detectable molecules. Consequently, it is clear that high-sensitivity detection is essential when performing any kind of analysis on a small scale.

Small volume detection in analytical technology has generally been based around optical measurements (either absorption or fluorescence). Unfortunately, small volume absorption measurements are compromised due to the difficulty in probing small volume cells, while maintaining a sufficiently long pathlength [47]. In microfabricated devices this problem is exacerbated (due to reduced feature dimensions), and to date, fluorescence methods have proved far more useful. However, some examples of absorption based detection have been reported. Absorbance detectors have been manufactured for whole column imaging and single point detection. The first was a single point UV-visible cell consisting of two chips (the lower one containing the fluorescence cell and the upper two windows for light introduction and collection) [48, 49]. A UV-cell with a longitudinal path subsequently provided a 10-fold increase in detection limits [47]. Photodiodes have been successfully used to detect changes in intensity due to a passing front of a chemical reaction [50]. Whole channel imaging has been adopted for use in micro capillary isoelectric focusing giving fast separation and detection times [51].

As stated, fluorescence based protocols have been used most widely for detection in chip-based systems. Detection limits for fluorescence based measurements are extremely low, and demonstrations of single molecule detection 'on-chip' have been reported [52–54]. Although fluorescence techniques are inherently sensitive, they are costly and not applicable for all molecular systems (i.e. not all species that absorb radiation fluorescence). Fluorescence protocols have been used to detect a variety of species including dyes [55], oligonucleotides [54, 56–60], double stranded DNA [34, 61], amino acids [48, 49, 57, 62–69] and metal ions [70, 71].

A recent innovation in optical detection is the development of Shah Convolution Fourier Transform Detection by Crabtree and co-workers [72]. This technique uses a novel convolution-detection method to convert multiple-point detection, time-domain electropherograms to frequency-domain plots. Analytes are detected on the basis of their 'blinking' frequencies, and although resolution in initial experiments was inferior to that seen in conventional single point detection, optimization of chip design, light collection and mathematical algorithms should lead to a performance similar to that of most single-point techniques. In another development, Eijkel et al. have recently

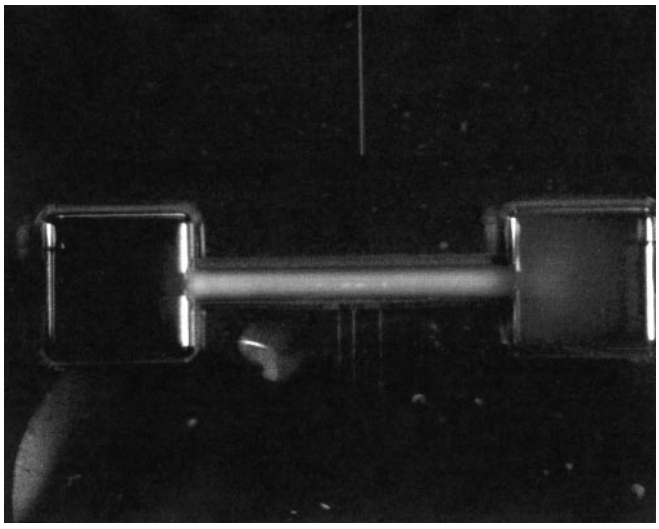

Fig. 3 Plasma burning in a $2000 \times 350 \times 150$ μm (105 nL) chamber in 75 Torr helium. (Courtesy of Dr. J. C. T. Eijkel)

presented a molecular emission detector on a chip that employs a direct current helium plasma for molecular fragmentation and excitation [73]. The detection limit for methane with such a device was 500 ppm. However, the lifetime of this detector was limited to just 2 h. Further research should increase the device lifetime and make it a more viable option for applications such as gas chromatography. Figure 3 illustrates a false-color image of a typical helium plasma burning within the microfabricated device.

Indirect fluorescence protocols have been used to detect compounds that are not fluorescent [74, 75]. In capillary electrophoresis, a fluorescing anion used in the buffer generates a large fluorescence background signal at the detector at all times. When analyte molecules enter the detection volume, the fluorescence background signal is lowered, because solute ions displace fluorescent buffer ions. Sirichai and co-workers have recently used this approach for the on-chip, electrophoretic analysis of a photographic color developer [76].

Other optical techniques in chip-based analysis systems include electrochemiluminescence [77, 78], and chemiluminescence [79]. Both approaches offer high sensitivity due to the absence of an optical source for excitation, but are limited in their applicability. In addition, Burgraff and co-workers have demonstrated the feasibility of refractive index detection on chip [80]. Holographic optical element (HOE) was used for measuring changes in refractive index in electrophoretic separation and analysis of carbohydrates on-chip.

Non-optical detection protocols have recently been applied to chip-based analytical systems. Advantages of these techniques include high sensitivities and wider applicability. Two examples are discussed here. Mass spectrometry (MS) is a fingerprinting technique of wide applicability and high sensitivity. MS needs minimal sample preparation, allows structure elucidation and accurate

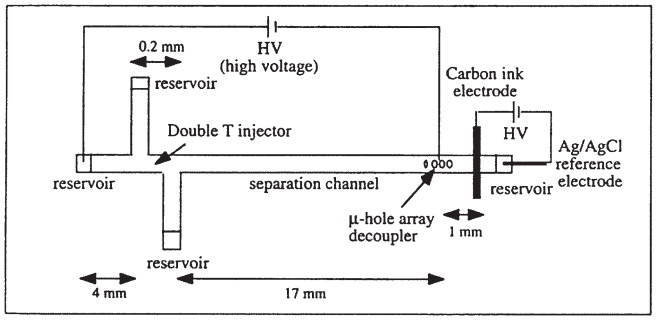

Fig. 4 A schematic of a microchip employing amperometric electrochemical detection. The reference electrode (Ag/AgCl) was situated outside the channel in the reservoir, whilst the two carbon working and counter electrodes were situated within the microchannel (Reprinted with permission from [89])

molecular weight determination. Furthermore, DNA based assays are easy to perform. The first microfabricated glass chip coupled to an MS detector used electrospray ionization (ESI-MS). Hormone samples were pumped by pressure and aligned by a precision translation stage to the MS inlet [81]. Ion trap MS [82] and ESI-MS [83] have also been used with electroosmotic pumping for the analysis of protein samples. A separation followed by MS detection was carried out by Karger and co-workers [81]. Coupling of MALDI-TOFMS to the MATCI (*m*iniaturized *a*nalytical *t*hermal *c*ycling *i*nstrument) described in Section 5.2.3 allows for complete PCR and product analysis in 50 min [84]. Recent research has concentrated on cleanup procedures for complex biological samples in ESI-MS [85–87]. For example, Smith and colleagues have integrated microdialysis and an ESI injector for MS [86].

Electrochemical detection provides a promising approach to miniaturized, high-sensitivity analyses. Woolley et al. have shown the feasibility of electrochemical detectors on microfabricated capillary electrophoresis chips [88]. Using indirect electrochemical detection they performed both DNA restriction fragment and PCR product sizing in ultra-short times. A schematic diagram of a planar glass CE chip with the integrated electrochemical detector is shown in Fig. 4 [89].

4.3 Reactors and mixers

As described in Section 2.2, the size of features in micromachined devices generally restricts fluidic mixing to diffusion. In large channels (> 100 μm), mixing of two components can take a long time (seconds to minutes). Consequently, micromixer design is a non-trivial process. Efficient mixing in the laminar flow region usually uses one of the following principles: (1) Elongated flow or laminar shear deforms or stretches fluid elements to generate increased interfacial mixing, (2) distributive mixing physically splits the fluid streams into smaller segments and redistributes them in such a way that the striation thickness is significantly reduced. This can be achieved by relative motion between the streamlines or changes in flow channel geometry. A number of publications describe fluid mixing or the lack thereof in microstructures [90, 91]. Examples of a few micromixer designs are now presented.

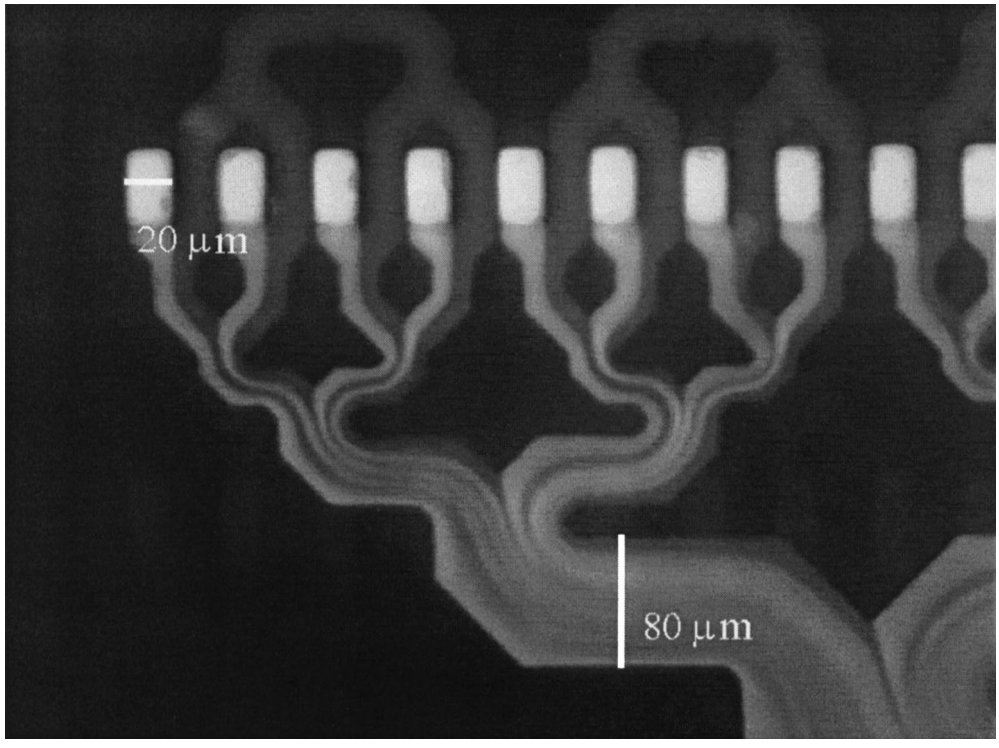

Fig. 5 Visualization of lamination and flow patterns formed when mixing fluorescein and rhodamine B in a silicon/glass micromixer (flow rate of 50 μL/min). The smallest channels are 20 μm wide, while channel depths vary between 49 and 58 μm. (Courtesy of F. G. Bessoth)

An elegant micromixer described by Blankenstein and Larsen splits the components into many smaller channels, thereby enhancing the diffusion of the two solutions [92]. Two other simple micromixers based on this design have also been presented [93]. Mixing times on these devices is of the order of 1 s. In addition, a Möbius-type mixer has been presented by Schwesinger et al. [94]. Bessoth et al. have designed and fabricated a micromixer (Fig. 5) that drastically reduces the time required for mixing two components using a multichannel split-and-mix concept [24]. The logic of this device relies on the fact that as one component is split into *n* smaller channels, the rate of diffusive mixing increases by a factor of n^2. Thus, splitting a channel into eight equally sized smaller channels allows the diffusion of a second component to occur 64 times faster. Typical mixing rates were on the order of 50 ms. Finally, mixing in some microstructures can occur via turbulent mixing (high Reynolds number regimes). For example, a mixing device consisting of two 'T' shaped channels has been reported to rely on turbulent flow, and shown to quench a base hydrolysis in 110 μs [95].

4.4 Filters

The use of integrated filtration systems on chip has only recently been investigated. For obvious reasons (i.e. the analysis of 'real' samples), the need for in-line filters on microdevices is great. Filters investigated range from mesh-type filters to dialysis membranes [42, 96–99]. The majority of these devices have dealt with filtration of blood or blood products. One method used to separate different cell types in blood is lateral percolation [96]. The filter excludes particles as small as a few microns. A similar method can be used to monitor erythrocytes [98] and to separate white blood cells from red blood cells [100].

Dialysis membranes have been used to separate plasma from whole blood [99], desalt DNA and protein sample prior to mass spectral analysis [42], and selectively remove amino acids from a homogenized tissue sample [97]. An in-line microfabricated filter has also been developed [101]. A range of fluid filters that discriminate on the basis of diffusion can also be used to separate molecules [102]. These methods rely on differential diffusion rates, based on molecular size, to preferentially enrich one outlet channel with the desired product.

4.5 Pumps and valves

A large proportion of microfluidic systems research has focused on the use of capillary electrophoresis (CE) on chip. This is primarily due to the fact that electrokinetic effects provide both an efficient transport mechanism and a simple way to control fluid flow in an interconnected network of channels. Early mechanical pumps could not produce the low, pulse-free flow rates needed on microchips. The need for pumps that could achieve steady flow rates and be small enough to be placed on the chip was apparent. Pumps have now been fabricated through photolithographic methods that produce nL/min flow rates [34]. For example, Ford et al. [34] have designed a pump consisting of a piezoelectric actuator, a pivoted lever, and a micro-syringe that yields flow rates of 1 nL/min. Wilson and colleagues have developed a positive displacement micropump for use with dialysis [103]. This pump, comprising of a polyimide diaphragm and two check valves, has a low dead volume and a reproducible flow rate ranging from 0.1 to 110 μL/min.

Burns et al. have described the manipulation of nanoliter sized droplets with a thermocapillary pump on microfabricated devices [104]. The concept relies on differential heating of the channel containing the droplet; heating the receding edge of the droplet decreases the surface tension and increases the internal pressure on the rear of the droplet, forcing the droplet towards the area of lower pressure. A second thermal pump has been described by Morrissey et al., where repeated heating causes the distortion of a machined silicon membrane, leading to the movement of fluid through the device [105]. In addition, Young has developed a micromachined vacuum pump that runs by the thermal transpiration effect and does not need valves to operate [106].

Another important, non-mechanical mechanism for pumping fluids is electrohydrodynamic (EHD) flow [107]. EHD pumping uses an electric field to induce a travelling wave along a microchannel. This in turn causes the movement of bulk liquid. Electrodes are regularly spaced along a channel and each electrode is phase shifted (rectangular voltage) with respect to its nearest neighbor [102, 107]. This method is limited to fluids that are dielectric and of low conductivity [102]. EHD flow is similar to dielectrophoresis (DEP), which uses similar principles to move particles [108].

Further development of the microchip has led to growth in the area of micromachined valves. There are two general types of valves: active and passive [102]. Active valves are those with an actuator (e.g. piezoelectric, coil spring, or thermopneumatic), whereas those without an actuator are passive valves (and usually are used as check valves with micropumps) [102]. Koch and coworkers have designed a cantilever valve to be used in conjunction with a membrane-actuated micropump [109, 110]. The 70 μm thick membrane pump consists of a thin wafer with a piezoelectric plate above it that causes the pumping action. The pump has flow rates between 490 μL/min for 4 kHz and 220 μL/min for 34 kHz at 240 V. The two cantilever (one way) valves are micromachined and placed at the inlet and outlet of the pump [111]. These ensure that the liquid flows in a single direction. Acera et al. have fabricated a similar micropump with two valves [112], whereas Carrozza et al. have made and tested a piezoelectric pump consisting of two ball (or check) valves [113]. In an elegant study, Vandelli and coworkers developed an array of active microvalves to control the flow of fluid through a micromachined device [114]. By controlling each valve independently, the flow rate through the device varied linearly with the number of valves open.

A valve that does not possess any moving parts and has zero dead volume is the freeze/thaw valve (FTV) [59]. The valve works, as the name suggests, by freezing a small plug of liquid in a channel. This prevents further flow; subsequent thawing of the plug resumes normal flow. Unfortunately, the response time of this valve is 20–25 s on closing and 35–45 s on opening.

Ramsey et al. have produced a 'microfabricated injection valve' [115]. This 'valve' is in fact a porous polysilicate membrane in a channel that allows the passage of ionic current, but prevents the passage of large molecules such as DNA. Consequently, DNA can be concentrated at this plug. By switching the voltage, the concentrated DNA can be injected into a separation channel. This device should allow for the facile manipulation of very small amounts of DNA on microfabricated devices.

One exciting application of microfluidic devices is in the microdelivery of active compounds. Santini et al. have developed a device which releases several compounds without the use of moving parts [116]. The chip contains square reservoirs filled with desired compounds. The tops of these reservoirs are covered with a thin layer of gold (0.3 mm – check reference for correct size) and connected to an anode. When the gold layer is subjected to chloride ions and an electric potential, soluble gold chloride complexes form, releasing small, measured amounts of compounds from the reservoir. As each reservoir can be connected separately, many different species could be released at varying rates.

The advent of additional pumping mechanisms has not diminished the use of electrokinetic pumping and valves on microfabricated devices. As well as continuing to be used in CE instruments, EOF is used in flow injection analyzers [117, 118], mixers [95], micromachined electrospray devices [119, 120], and numerous other applications. The primary reason for this popularity is the fact that the devices are valveless, therefore reducing dead volumes and increasing analytical performance [57, 58, 67]. EOF remains a very reliable and efficient method for movement of liquid.

4.6 Other components

The need to direct specific sample populations in microfluidic devices has prompted the development of flow switches [92]. A typical flow switch consists of 3 inlets, a channel, and several outlets. Sample is introduced in the middle inlet and by adjusting the flow rates of the side inlets, sample can be directed into any of the outlets. This device can act as an in-line injection valve. A second micromachined injector takes the shape of a double-T design [121]. This layout allows the formation of 50–500 pL sample plugs for injection. A more primitive injection valve has been used to introduce sample from a PCR chamber to a CE device [122]. Using a viscous sieving matrix, DNA is electrokinetically introduced onto the CE device whilst the bulk of the reaction mixture remains in the PCR chamber. A recent example of a micromachined centrifuge is the LabCD™ from Gamera Biosciences [123, 124]. Here fluidic movement is caused by rotation of the compact disk (CD). Channels and other components are etched into a CD and samples are placed onto this platform for analysis.

5 Application areas

5.1 Separations

In the early 1990s most microfluidic research was focused on separation technologies, especially capillary electrophoresis (for reasons described in Section 4.5). Manz and colleagues reported the first on-chip CE separations in 1992. These showed the separation of fluorescein and calcein within 5 min on a planar glass chip [8, 125]. Many separations were subsequently performed in similar chip designs. These included small dye molecules [126] and labeled amino acids [48, 49, 62, 63, 66]. At the same time other groups developed similar techniques [55, 64, 67, 68].

In the 1990s, the separation of both long chain oligonucleotides and double stranded DNA was achieved in CE microchips [57, 58]. An example of a subsequent chip-based analysis of a DNA restriction digest (using a PDMS chip) is shown in Fig. 6 [54]. The feasibility of μ-TAS for DNA sequencing has also been demonstrated [59, 127, 128]. For example, Woolley et al. have performed DNA separations with single base pair resolution on microfabricated CE chips. Sequencing extension fragments were separated to 433 bases within 600 s using a one-color detection protocol and a separation length of 35 mm [128].

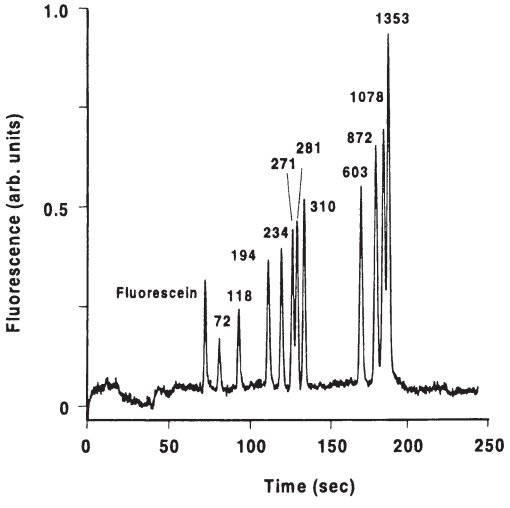

Fig. 6 The electrophoretic separation of a ϕX-174/HaeIII DNA restriction fragment digest on a PDMS microchip. L = 35 mm; E = 500 V/cm; injection volume = 150 pL; amount injected per fragment = 55 zmol. DNA concentration is 1.3 ng/μL in Tris/boric acid buffer (Reprinted with permission from Effenhauser CS, Bruin GJM, Paulus A, Ehrat M (1997) Anal Chem 69:3451–3457. Copyright 1997 American Chemical Society) [54]

Other techniques that have been miniaturized and transferred to chip-based formats include synchronized cyclic capillary electrophoresis [48, 129], free flow electrophoresis [130], micellar electrokinetic capillary chromatography [131], microchip liquid chromatography [132], packed-bed liquid chromatography [133], and open channel electrochromatography [132]. Separations continue to be a part of ongoing research in microfluidics [134, 135].

5.2 Reactions

5.2.1 Derivatization reactions

The next stage of development involved the integration of a separation and reaction stage on-chip. Early examples from Fluri et al. and Ramsey and co-workers included simple pre- and post-column reactions (including derivatization reactions, and restriction enzyme digests) [65, 69, 136]. In addition, Mangru [79] and Eijkel [137] have presented reactors for derivatization of samples. Derivatization reactions are clearly important in chemical separations and have been used for many years in bench-top systems.

5.2.2 Enzymatic reactions

An important biological use of microchips is the miniaturization of enzyme reactors. Initial examples have focused on systems using glucose oxidase. These devices measure glucose concentration by covalently immobilizing glucose oxidase to the walls of the chip. In early examples V-shaped channels micromachined in silicon were used [138, 139]. The advantages of both miniaturization and the V-shaped channel are linked to the thin layer of immobilized enzyme. A thin layer provides a higher ratio of effective enzyme to total enzyme. This means there is no meaningful diffusion of substrate into deeper enzyme layers, thus ensuring the reactor is flow independent. However, V-shaped channels still have the limitation of low surface area defined by the V-groove geometry and planar channel walls. High aspect ratio, vertical channels have proved more successful [21, 140, 141] and the use of a porous silicon surface layer has been shown to increase efficiency by two orders of magnitude [22, 142]. Similar enzyme reactors (using immobilized peroxidase) have also been developed to measure hydrogen peroxide [138, 140, 143].

5.2.3 Integrated DNA analysis

As this decade comes to a close, more and more μ-TAS devices are being developed for biological purposes, specifically DNA analysis. This has been driven, in large part, by the Human Genome Project, and as described previously, the analysis of DNA is one of the most dominant application areas for μ-TAS. Much initial research focused on making single components (e.g. reactors, mixers, and separation systems) on chip. Fabricating a fully integrated device, however, would allow for high-throughput biological analyses to take place on a large-scale [144]. Integrated devices can be envisioned as a single unit or as smaller interchangeable 'building blocks' that can be connected together [145]. Harrison [47, 79, 121], Ramsey [56, 69, 136], Mathies [88, 122], and Effenhauser [54] have all combined CE with other components on chip. Post-column reactors [69, 79, 136], restriction enzyme reactions [54, 56], electrochemical detection [88], an injector [121], and PCR [122] have all been successfully integrated with CE on chip.

An early demonstration of integrated DNA analysis, by Ramsey and co-workers, described an integrated restriction fragment analysis on a glass microchip. A DNA sample was mixed with the restriction enzyme in a dilated microchannel and electrophoretic analysis used to determine fragment distribution of the resulting digest [56]. Demonstrations of more complex on-chip reactions involving DNA have tended to focus on reactions such as the Polymerase Chain Reaction (PCR) [122, 146].

PCR is a very important tool in molecular biology. It allows any nucleic acid sequence to be generated in-vitro, in abundance. Its theory and mechanism have been discussed extensively elsewhere [147]. Conventional thermal cycling machines used to perform PCR generally require long cycling times due to large time constants associated with heating and cooling. However, system miniaturization allows the use of very small sample volumes (nL) and materials with high thermal conductivities such as glass or silicon. Both characteristics allow cycle times to be drastically reduced.

Early microfabricated PCR devices reported considerably faster cycling speeds than conventional instrumentation [104, 148, 149]. These systems have performed PCR in small volume (4–12 μL) silicon/glass reaction chambers placed in larger conventional thermal cyclers [148, 150], 20–50 μL microfabricated silicon chambers with integrated heaters and temperature control [151, 152], and on a 25 μL drop of solution above a microfabricated heater [104].

Development of the microdevice described in [151, 152] has recently yielded a portable device that performs PCR with real-time detection of reaction products [84, 153–155]. The *m*iniaturized *a*nalytical *t*hermal *c*ycling *i*nstrument (MATCI) uses microfabricated PCR chambers in conjunction with a light emitting diode source for real-time fluorescence detection. Complete sample analysis could be performed within 10 min [153]. In addition, further studies demonstrated efficient detection of single nucleotide polymorphisms from human C6 gene samples [154]. A second generation MATCI system (the Advanced Nucleic Acid Analyzer, or ANAA) has since been produced [156, 157]. In this system an array of 10 chambers allows for parallel sample analysis.

At a higher level of sophistication, Woolley and coworkers presented the integration of ultra-fast PCR and

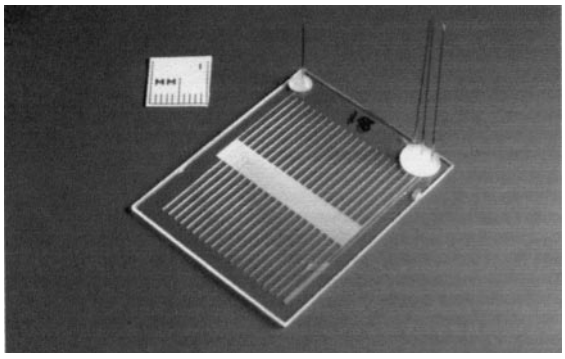

Fig. 7 Photograph of the continuous flow PCR chip. The chip is fabricated in Corning 0211 glass and all channels are 90 μm wide and 40 μm deep. Access to channels is facilitated by 400 μm holes drilled into the cover plate. (Courtesy A. J. de Mello)

DNA sizing on a single microdevice [122]. In this study the PCR chamber described above [151, 152] was integrated with a planar CE microchip. Subsequently, a 268 base-pair product from the β-globin gene was amplified and sized by CE within 20 min of sample introduction. Furthermore, the authors described a rapid assay to identify genomic Salmonella DNA in less than 45 min. This technology clearly demonstrates the potential of integrated μ-TAS in future diagnostic applications. In a similar approach, Ramsey et al. presented an integrated device for cell lysis, PCR and electrophoretic sizing. In this case cycling was achieved by placing the entire chip within a conventional thermal cycler [158, 159].

An alternative and exciting approach to PCR (and chemical reactions in general) has been developed by Kopp and co-workers (Fig. 7). The novel use of a continuous flow microchip allowed heating and cooling times on a millisecond time scale, and performed amplification of the Neisseria gonorrhoeae DNA gyrase gene in times as short as 90 s [146].

Other noteworthy achievements in the field of miniaturized PCR include the isolation and PCR of DNA from white blood cells [100] and degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR) with CE sizing of products [160]. Furthermore, Burns et al. have recently described a device that integrates a nanoliter injector, mixer, temperature controlled reaction chamber, an electrophoretic separation system and a fluorescence-based detector on single chip [104, 161]. DNA digestion and amplification can both be performed. Importantly, almost all components used in this chip were micromachined on a single glass and silicon substrate through photolithography. This allows the fabrication process to remain simple and applicable to mass production.

5.2.4 Other biological applications

Cell sorting. Many early chip studies applied to biological applications centered on the analysis of biological parameters, e.g. blood [23] and blood gases [162–164]. Furthermore, whole cell chips can be used to measure extracellular fluid to monitor intracellular processes, and in evaluation of new drugs [165]. More recent work has centered on the manipulation of complete cells on chip [166–169].

Pethig and co-workers are using dielectrophoresis (DEP) to manipulate cells on planar chips [108, 170, 171]. Movement by dielectric polarization is achieved through the use of non-uniform electric fields [171]. Movement of large numbers of cells can be effected using numerous offset electrodes on either side of a channel. If the phase of each electrode varies by 90° with respect to neighboring electrodes, a travelling electric wave is created. This in turn interacts with the charged membranes of cells and causes motion [108]. In addition, strips of electrodes placed orthogonal to each other on the top and bottom of a channel allow single cells to be individually moved [170]. A similar method has been developed by Fuhr and colleagues [168, 169]. DEP has also been used to separate *E. coli* from a mixture followed by lysis and DNA analysis [172]. Wilding et al. have taken this idea one step further and added PCR analysis after the cell lysis [100].

Diagnostic applications. Systems for biological and medical analysis have also been developed including micromachined sensors for PO_2, PCO_2 and pH for online blood monitoring [163, 164], and a silicon device for detecting the metabolic activity of living cells [173].

6 Commercial exploitation

The number of companies now involved in biological microsystem technologies is evident from two recent conferences, Nanotech 98 and μ-TAS 98. Over 75 companies and organizations from North America, Japan, and Europe, directly involved in microsystem research, participated in Nanotech 98, while over 40 companies and many other organizations were involved in μ-TAS 98. Importantly, several strategic alliances have been formed over the last two years. These include, Caliper Technologies Corporation and Hewlett-Packard/Agilent Technologies, Orchid Biocomputers and Advanced BioAnalytical Services, and Perkin-Elmer and ACLARA BioSciences [174]. Cellomics have also announced an alliance with Carl Zeiss and are collaborating with both Warner-Lambert and Merck [174]. Table 1 provides a representative list of companies involved in microfluidics together with their area of research.

Leading companies in chip based technologies include Nanogen, Affymetrix, Caliper Technologies, and Orchid Biocomputers and Gamera Biosciences. Nanogen is working towards an electronically mediated, disposable DNA array microchip [184]. Arrays of oligonucleotides are covalently bound to specific sites on the chip through ‘electronic addressing’. Negatively charged DNA, when introduced, is drawn to positively charged sites. Chips of this type have been used to separate carcinoma cells from peripheral blood cells [167]. When combined with on-chip cell lysis, a highly integrated diagnostic device should result [167].

Another long-standing company in the microchip field is Affymetrix. They are developing the GeneChip® Information Systems which provides efficient access to genetic information using miniaturized, high-density arrays of oligonucleotide probes [175]. This is geared to studies in expression analysis, genotyping, and disease management. Light Directed Combinatorial Synthesis is used to fabricate the chip array [199, 200]. Hybridization data are collected as light emitted from fluorescent reporter groups already incorporated into the target. Probes that perfectly match the target produce stronger signals than those having mismatches. Since the sequence and position of each probe on the array are known, by complementarity the identity of the target nucleic acid applied to the probe array can be determined.

In September 1999, Caliper Technologies announced the launch of the first LabChip™ technology-based microfluidic instrument [181]. The desktop-size instrument, the Agilent 2100 Bioanalyzer (formerly Hewlett-Packard), is designed to perform nucleic acid analysis using microfluidic chips. Sample manipulation is controlled through electroosmotic pumping and chips are disposable.

Finally, Orchid Biocomputer has developed the reusable ChemTel™ chip [179]. These chips are able to process hundreds of reactions in parallel through the use of precise fluidic delivery methods. A matrix of non-mechanical microvalves is fabricated within the multi-layered chip to control both vertical and lateral fluidic management, without any risk of evaporation or reagent degradation. Recently, the company has introduced a chip based on genetic bit analysis (GBA), and has used it to detect the Mycobacterium tuberculosis rifampin resistance mutation [201]. In collaboration with the bioinformatics program at Cold Spring Harbor Laboratory, Orchid will implement high-throughput methods using GBA technology to assay and confirm the authenticity and location of genetic markers called single nucleotide polymorphisms (or SNPs).

7 Conclusions and outlook

As is evident from this brief review, the last decade has been a time of rapid technological and scientific development for μ-TAS. More specifically, the theoretical predictions (of increased efficiencies, speeds and throughput) have in general been borne out by experiment. Initial 'proof-of-principle' devices relating to single components of a complete analysis have been demonstrated and now the challenge is to integrate these on single microdevices. This challenge is being addressed, and the degree of functional integration is steadily increasing. Perhaps the area of most current interest lies in successful sample handling and pre-treatment. Although sample manipulation via electrokinetic and hydrostatic mechanisms is widely understood, analysis of 'real' samples will require more sophisticated infrastructure. Success in this specific area will determine many of the eventual applications for microfluidic chips.

Over the past five years many new materials and fabrication methods have been introduced. Of these, the use of

Table 1 Companies involved in microfluidic research (Note: the authors do not endorse any of the companies or products)

Company	Research	Reference
Affymetrix	Genetic analysis	[175]
Tomtec Inc.	High throughput screening	[176]
MicroParts GmbH	Micromolding for medical applications	[177]
Jenoptik Mikrotechnik	Hot embossing microfabrication	[178]
Orchid Biocomputer Inc.	Solid phase organic synthesis	[179]
Gene Logic Inc.	Drug discovery and gene expression	[180]
Caliper Technologies	Clinical laboratory diagnostics, DNA analysis	[181]
Micronics Inc.	Clinical laboratory diagnostics and analytical and process control	[182]
Sequenom Industrial Genomics	DNA array and Mass Spectrum detection	[183]
Nanogen Inc.	DNA arrays	[184]
Cepheid	DNA analysis	[185]
Incyte Pharmaceuticals, Inc.	DNA microarrays	[186]
EVOTEC Biosystems AG	Drug discovery	[187]
Genometrix Inc.	High throughput microarrays	[188]
Symyx Technologies Inc.	Combinatorial chemistry	[189]
Cellomics, Inc.	Drug discovery	[190]
Rikilt-DLO	Agricultural quality control	[191]
Genetic MicroSystems Inc.	Genomic research and drug discovery	[192]
Trega Biosciences Inc.	Solid phase organic synthesis and drug screening	[193]
ALCARA BioSciences	DNA sequencing	[194]
Alexion Pharmaceuticals Inc.	High throughput screening	
Packard BioScience Inc.	Massively parallel DNA array analysis	[195]
H. Weidmann AG Plastic	Injection molding of microchips	[196]
Technology Innogenetics	DNA analysis for genetic diseases	[197]
Dynal AS	Magnetic polymer particles for biomagnetic separation	[198]

polymeric substrates is particularly interesting. Furthermore, the miniaturization of associated control architecture (e.g. pumps, filters, detectors and power supplies) will allow the creation of truly miniaturized, integrated systems for 'in-the-field' and 'point-of-care' applications.

As stated, the generality of the μ-TAS concept makes it applicable to many areas of modern day analysis. Interest from both academic and commercial sectors is now at a significant level, evidenced by both corporate and governmental spending on chip-based programs. Research over the past decade has demonstrated many of the potentials of microfluidic chips for chemical and biochemical applications, however, the next 5–10 years will almost certainly define the timeframe when the true applications of μ-TAS are realized.

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