Chapter 2

Chip Capillary Electrophoresis and Total Genetic Analysis Systems

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Abstract

The utilization of new sequencing techniques based on capillary array electrophoresis (CAE) has had a great impact on the progress of the Human Genome Project (HGP), and finally led to its successful completion at much lower costs than initially anticipated for the project (Collins *et al.*, 2003). Similarly, chip-based capillary electrophoresis, the technological extension of capillary electrophoresis (CE), is a rapidly emerging technology, which has caused revolution in analytical chemistry. In fact, there has been an explosion of interest in the development of chip-based CE ever since the initial concept "micro-total analysis systems (μ-TAS)" or "lab-on-a-chip" was introduced by Manz and Harrison (Manz *et al.*, 1990; Harrison *et al.*, 1993). With great efforts from leading scientists, this new-born technology has matured rapidly. It has several advantages over conventional methods, such as reduced analysis time, high efficiency, low sample consumption, the potential for integration and automation, disposability, portability and so on. All these features make chip-based CE an attractive technology for the next generation of CE instrumentation. For example, a binary mixture could be successfully resolved in 0.8 ms using chip-based CE separation using a field strength of 53 kV/cm, with an analysis time of several orders of magnitudes less than conventional CE (Jacobson *et al.*, 1998). Other groups have applied chip-based CE for separating FITC-labeled amino acids, and the plate heights obtained could be down to 0.3 μm, which demonstrated the high efficiency of this separation technology (Effenhauser *et al.*, 1993). More recently, a microfabricated 384-lane CAE device has been developed and used for highly parallel genetic analysis, showing great promise as a means for ultra high-throughput bioanalysis (Emrich *et al.*, 2002; Paegel *et al.*, 2003). With the maturation of these technologies, companies have initiated industrialization of chip-based CE products. Several commercial chip products are currently available, such as the 5100 Automated Lab-on-a-chip Platform from Agilent Co., Ltd., and the Labchip 90 Electrophoresis System from Caliper Co., Ltd. This chapter will give an overview of the chip-based CE technology, including microchip design, fabrication and detection, surface modification and applications.

1. INTRODUCTION

1.1. Various chip-based capillary electrophoresis systems

Chip-based CE is based on the microfabrication techniques developed in the semiconductor industry, with microchannels of a desired pattern for the

particular application fabricated using photolithography or micromolding processes. These techniques are used to produce both the microchannel separation device and attendant structures, and to integrate electrodes, detection apparatus and other electro-mechanical control devices needed for the operation of the microchannel device. Manufacture will be discussed in detail in Section 3. For a typical electrophoretic separation experiment on a microchip several steps are involved. First, all channels should be filled with running buffer or sieving matrix. After the sample is loaded into a sample reservoir, it is transferred electrokinetically into an injector region, forming a sharp band. Then, a high voltage is applied and the sharp sample band is forced into the separation channel, and it begins to separate according to the properties of the separating environment and media. When each of the resolving species reaches the detection point at the end of the separation channel, signals are produced and recorded to form an electropherogram. The above-mentioned steps are typical for capillary zone electrophoresis (CZE), which is the first electrophoretic format transferred from conventional CE onto microchips. In this format, all samples are electrophoresed in free solution, and separated into zones according to their charge-to-mass ratio. There are other formats besides CZE for chip-based CE, formats such as capillary gel electrophoresis (CGE), isoelectric focusing (IEF), micellar electrokinetic capillary chromatography (MECC), isotachophoresis (ITP), capillary electrochromatography (CEC), etc. A brief introduction to microchips using these different electrophoretic formats are given below.

CGE is a most widely used format for chip-based CE. Microchannels are filled with sieving matrix, which is cross-linked gel or entangled polymer solution, instead of a free solution. The analytes electrophoresed in this media are separated according to their size. DNA fragment sizing, genotyping and sequencing have been demonstrated with chip-based CGE (Effenhauser *et al.*, 1994; Schmalzing *et al.*, 1997; Shi and Anderson, 2003).

IEF is a kind of electrophoresis in a pH gradient set up between a cathode and anode, with the cathode at a higher pH than the anode. During IEF electrophoresis, amphoteric samples (such as proteins) are separated according to their isoelectric point (pI) values, and at the end of the electrophoresis, each species migrate and concentrate at their isoelectric points. For chip-based IEF electrophoresis, which utilizes single-point detection system, forces are needed to mobilize the focused sample band to the detection point. The most common mobilization methods are chemical, hydrodynamic and electro-osmotic flow (EOF)-driven mobilization, among which EOF-driven mobilization proves most suitable for miniaturized systems because of its high speed and low instrumentation requirements (Hofmann *et al.*, 1999). Typically, systems use EOF to drive samples to a single-point for detection. However other systems such as whole column imaging detection have also been developed, such that the sample band mobilization step is eliminated (Mao and Pawliszyn, 1999a, 1999b).

MECC is another kind of electrophoresis that has been successfully transferred into microchip and is widely utilized. MECC is an operational mode of CE developed by Terabe *et al.* (1985) to address the general problem of CE of an inability to separate uncharged species. A surfactant such as sodium dodecyl sulfate (SDS) is added to the running buffer in sufficient concentration

to form micelles. In a typical run, the micelles move much more slowly toward the cathode than does the running buffer solution (driven by EOF). The partitioning of solute between the micelles and the running buffer provides a separation mechanism similar to that of liquid chromatography. Separation of various samples such as neutral coumarin dyes (Moore *et al.*, 1995), amino acids (Rodriguez *et al.*, 1999) and explosives (Wallenborg and Bailey, 2000), has been successfully demonstrated using microchip MECC, showing enhanced separating efficiency and decreased analysis time compared to conventional MECC.

For ITP, two different buffer systems are used to create zones into which the analytes separate. For example, to separate anions a leading electrolyte is chosen whose anionic component has a higher mobility than that of the analytes. It will migrate faster than the analyte in an electric field. Similarly, a trailing electrolyte is also chosen with an anionic component that migrates slower than the analytes. When the electric field is applied the anions start to migrate, and the analytes will separate into zones determined by their mobilities, with the fastest analyte moving behind the leading electrolyte. A demonstration of ITP on microchip was provided by Walker and Morris (1998), where they performed ITP separations of herbicides paraquat and diquat on a glass microchip, with an on-chip detection using normal Raman spectroscopy.

CEC is a hybrid separation method that couples the high separation efficiency of CZE with high-performance liquid chromatography (HPLC), and uses an electric field rather than hydraulic pressure to propel the mobile phase through the stationary phase. With this combination, samples with similar electrophoretic mobilities can be separated, even when they cannot be separated by CZE alone. An additional benefit of CEC compared to HPLC is the fact that the flow profile in a pressure-driven system is parabolic, whereas in an electrokinetically driven system it is plug like and therefore much more efficient. There are three types of CEC according to the formation of the stationary phase, open tubular CEC (OTCEC), packed column CEC and monolith column CEC. Transfer of each such technique to the microchip format has been successfully demonstrated by Jacobson *et al.* (1994a), Ceriotti *et al.* (2002) and He *et al.* (1998).

2. CHIP DESIGN AND FLUID MANIPULATION

2.1. Chip design

The design of microchips has undergone significant development, and it is the sophistication of these designed structures that exemplify the differences between microchip devices and conventional capillary devices. The first and simplest chip designs consisted of microchannels laid-out in cross geometries, with a straight separation channel intersected by a second channel for sample injection. Four reservoirs were positioned at the end of each channel, two for sample and background introduction, and the other two serving as waste reservoirs (see Figure 1A). With the maturation of computer-aided design (CAD)

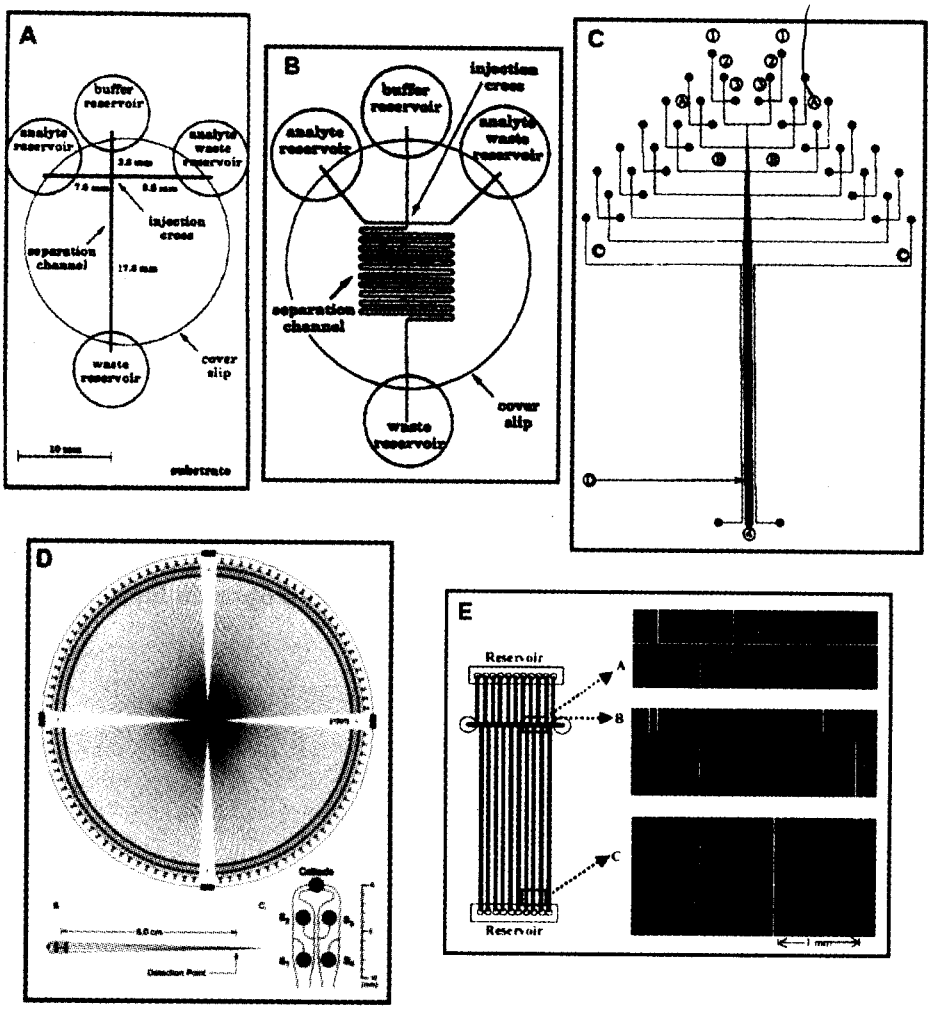


Fig. 1. Various designs for chip-based CE. (A) Microchip with a cross design, reprinted from Jacobson *et al.* (1994b) with permission; (B) microchip with serpentine turns in separation channel, reprinted from Jacobson *et al.* (1994c) with permission; (C) microchip with 12 channels, reprinted from Woolley *et al.* (1997) with permission; (D) microchip with 384-lane radial capillary array, reprinted from Emrich *et al.* (2002) with permission; and (E) microchip design for 2-D electrophoresis, reprinted from Li *et al.* (2004) with permission.

tools and microfabrication techniques more complex microchip designs have been developed, with features such as longer separation channels, serpentine turns, tapered turns, multichannels and capillary arrays for high-throughput analysis, two-dimensional electrophoresis, and others (see Figure 1B–E). With various improvements in microchip designs to overcome particular limitations, electrophoretic analysis with higher efficiency and throughput can be achieved, which greatly enhances the uses of chip-based CE for different types of analytical functions.

2.2. Fluid manipulation

Sample injection is a major concern of fluid manipulation on microchip. So far, three different injection methodologies have been established with chip-based CE: unpinched injection (Wang *et al.*, 1999a, 1999b; Martin *et al.*, 2000), pinched injection (Woolley *et al.*, 1998; Evans, 1997) and gated injection (Liu *et al.*, 2000).

Unpinched injections are performed using a single power supply. First, a high voltage is applied between the sample and the waste reservoirs for a short time. The sample is electrokinetically introduced directly into the separation channel. After the injection is complete, a high voltage is applied to the buffer and the waste reservoirs, which initiates the separation. However, in this unpinched mode, in which no push-back voltages are applied, irreproducible and larger-than-normal sample bands may result (Figure 2A).

Pinched injections are now the most widely used mode for sample injection on microchip. During the pinched injection, samples are injected electrokinetically into a volume-defined injector (cross or double-T shaped) with a pinching voltages applied at both the buffer and the waste reservoirs to define the sample band size. With this pinching voltage, short injection plugs with reproducibility

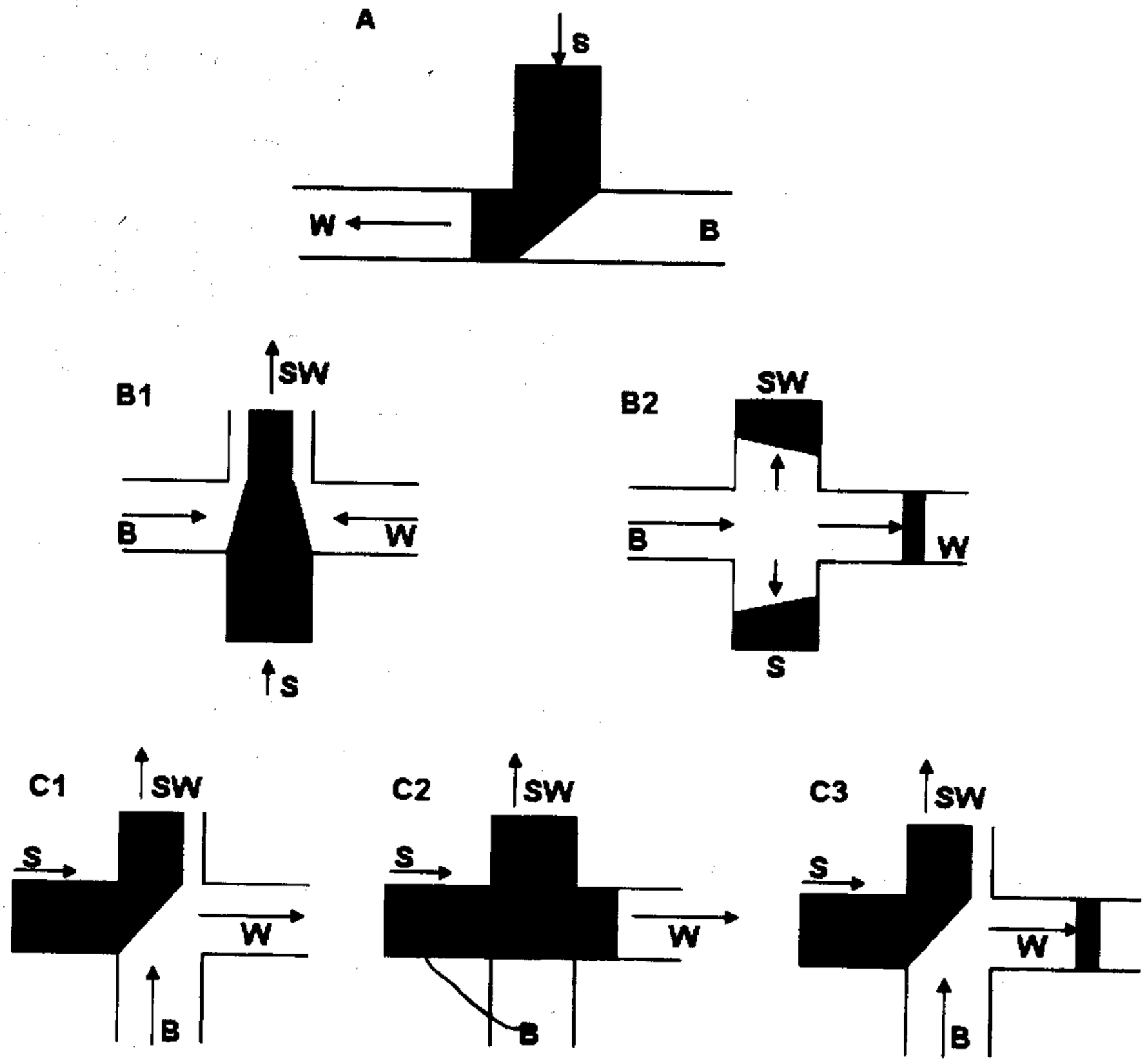


Fig. 2. Schematic views for thee types of injection methodologies. (A) Unpinched injection; (B) pinched injection; and (C) gated injection. Annotations: B, buffer reservoir; S, sample reservoir; SW, sample waste reservoir; and W, waste reservoir.

of peak heights better than 4.1% could be achieved (Evans, 1997). After the injection is complete, a high voltage is applied between the buffer and the waste reservoirs to initiate the separation, with a lower voltage applied at the injection channel to prevent the samples remaining in the injection channel from leaking into the separation channel (Figure 2B). With the pinched injection mode, the electrokinetic bias of the sample can be eliminated.

Gated injection is another widely utilized methodology for sample injection. With the gated injection scheme, the sample is introduced at the head of the separation channel, and buffer is placed in one of the side reservoirs. A high voltage is applied to the buffer reservoir and a fraction of that high voltage is applied to the sample reservoir with the sample waste and waste reservoirs grounded. This results in a flow of sample toward the sample waste reservoirs and a separation flow from the buffer to waste reservoir. Owing to the control of voltage, no sample will mix into the separation channel. To inject a sample plug into the separation channel, the high voltage applied to the buffer reservoir is removed for a short-time period (normally several seconds). Later, the separation step is initiated by resuming the high voltage to the buffer reservoir. This gated injection mode is very convenient to control the amount of sample plug injected into the separation channel. However, electrokinetic bias among samples cannot be avoided with gated injection mode (Figure 2C).

There are also other concerns with fluid manipulation on microchip, including the mixing of sample with labeling reagent or buffer (Kutter *et al.*, 1997), and methods for redirecting the sample after separation for collection of the separated fractions of the analyte (Effenhauser *et al.*, 1995). Successful fluid manipulation could be achieved only if chip design and voltage control are thoroughly studied and understood.

3. MATERIALS AND FABRICATION

3.1. Materials

Silicon is excluded as a material for fabricating CE microchips because of its semiconductivity proves problematic when high voltage is applied (Kopp *et al.*, 1997). Instead, various glass substrates are widely used, from inexpensive soda lime glass through high quality quartz. These substrates are chosen because of their good optical properties, well-understood surface characteristics, high efficiency in dissipating heat and well-developed microfabrication techniques transferred from semiconductor industry (Jacobson *et al.*, 1994d; Fan and Harrison, 1994). However, there are some disadvantages of using glass substrates as microchip materials that hinder the ongoing production and commercialization of glass chip-based CE devices, including the high cost of substrate materials, the many steps and harmful wet chemistry involved in microetching and limitations in geometrical designs available due to the isotropicity of the etching process. Researchers and industry however have explored the use of wide variety of polymer materials for fabricating microchips instead of glass,

Table 1. The properties of common fabrication materials used for microchips

Material property	Si (single crystal)	Glass	SiO_2	PDMS	PMMA	PC
Dielectric strength ($\times 10^6$ V/cm)	3	5–10	2–3	2.1	0.17–0.19	0.39
Coefficient of thermal expansion ($\times 10^{-6}$ C^{-1})	2.6	0.55	0.55	310	55	70.2
Thermal conductivity at 300 K (W/cm/K)	1.57	0.011	0.014	0.0018	0.002	0.002
> 70% optical transmittance (nm)	> 700	> 350	> 350	400–700	400–700	400–700
Maximum processing temperature (°C)	1415	550–600	1700	~150	~100	~100
Bulk resistivity (μΩ cm)	2.3×10^{11}	$> 10^{10}$	$> 10^{10}$	$> 10^{20}$	$> 10^{20}$	$> 10^{20}$
Temperature coefficient of resistance (10^{-3} K^{-1})	−70	—	—	—	—	—
Water contancy angle (advancing)	110°	20–35°	~30°	~110°	60–75°	78°
Glass temperature T_g (°C)	—	—	—	—	106	150

Source: Modified from Lagally and Mathies (2004) and Becker and Gärtner (2000) with permission.

including standard polymer materials. Some properties of these different materials are listed in Table 1.

These materials include polymers such as polyamide (PA), polybutyleneterephthalate (PBT), polycarbonate (PC), polyethylene (PE), polymethylmethacrylate (PMMA), polyoxymethylene (POM), polypropylene (PP), polyphenylene ether (PPE), polystyrene (PS), etc. To date, PMMA and PC are the most popular polymer materials for microfabrication employing hot embossing and injection molding techniques. Poly(dimethylsiloxane) (PDMS) is another very widely used polymer material for fabricating microfluidic devices because of extreme ease of fabrication and uniformity. A cycloolefin copolymer

(COC) is currently being tested for fabricating microchips, which shows very high promise for applications in chemical engineering and molecular biotechnology due to its high chemical stability and optically transparent properties.

3.2. Fabrication

3.2.1. Fabrication procedures for glass materials

Structures on glass substrates are usually generated using standard photolithography techniques (Woolley and Mathies, 1994). Briefly, a glass substrate is coated with a photoresist film. Then, transferring the channel pattern to the film is conducted by exposure to UV radiation through a patterning mask. The exposed portions of the film are dissolved, the remaining parts of the film are hardened by heating, and these then serve as a sacrificial layer for chemical etching. Wet etching using hydrofluoric acid is the most popular way for chemical etching of glass substrates. Finally, the etched substrate was thermally bonded to the top glass plate, which has access holes drilled into it. Figure 3 illustrates the procedure.

3.2.2. Fabrication procedures for polymer materials

Two main methods are utilized for microfabricating polymeric microchips according to the properties of polymer materials: replication technologies and

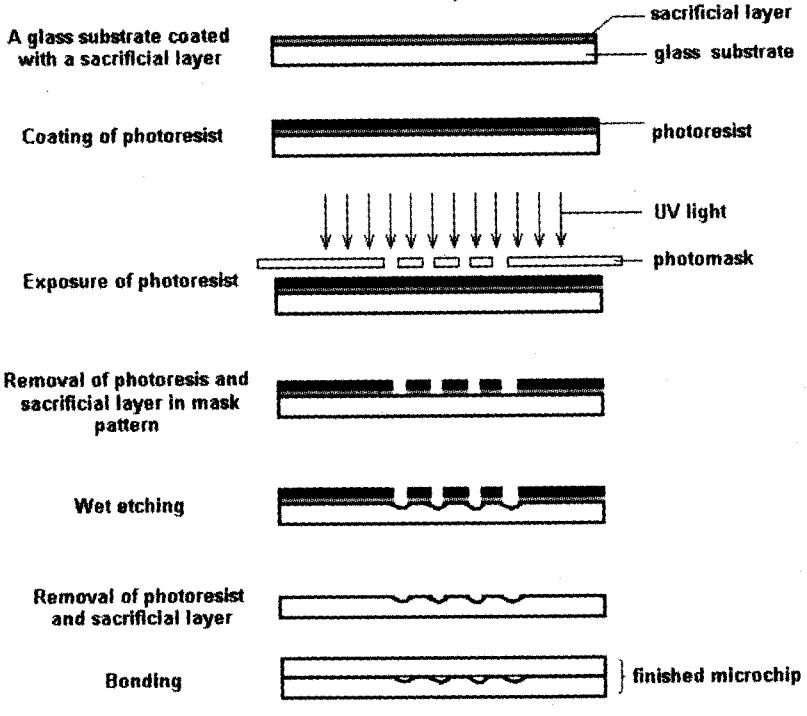


Fig. 3. Procedures for photolithographic fabrication with glass materials.

direct technologies. After the desired patterns are fabricated, a sealing process is needed to form the enclosed microchannels.

3.2.2.1. Replication technologies. The principles behind replication technologies are already well known in the macroworld fabrication. For example, injection molding represents a standard technology for macroscopic polymer component manufacturing. Thus, there is considerable interest to establish a low-cost manufacturing process in the fabrication of microchips using these technologies. However, there are several processes used with replication technologies that give concern for transfer to the microworld. First, undercuts (i.e. structures in the polymer with overhanging edges) cannot be fabricated since the master needs to be removed from the molded structures. Second, the surface quality of the master is a key factor for the lifetime of the mold tools and a limitation on the achievable aspect ratios. Typically, surface roughness values of better than 100 nm root mean square (RMS) are necessary for a good and reliable microstructure replication. Third, interactions between master materials and the polymer substrate should be prevented, as release agents are often used in the demolding step.

The key to the replication technologies is the fabrication of master molds. Generally, three methods are utilized for master fabrication, including micromachining methods (Martynova *et al.*, 1997), electroplating methods (Ehrfeld and Munchmeyer, 1991; Hesch *et al.*, 1995) and silicon micromachining methods (Jansen *et al.*, 1996). After master molds have been fabricated, several methods can be applied for the replication step, such as hot embossing, injection molding and casting.

Hot embossing is currently the most widely used replication process for the fabrication of microchannels onto microfluidic devices. A master with micropatterns is mounted in an embossing system together with a planar polymer substrate, both of which are heated in a vacuum chamber to a temperature just above the glass transition temperature of the polymer substrate. The vacuum is necessary to avoid bubble formation due to the entrapping of air in small cavities. Then, the master mold is brought into contact with the substrate and embossed with a controlled force for some period of time (typically several minutes). Later, the master-substrate is cooled to below T_g with force still applied. Diagrams of a hot embossing machine and a view of a microchannels fabricated using hot embossing are shown in Figure 4A and B.

Injection molding is another widely applied technology, with ability to form almost any geometry from a large variety of thermoplastic materials (McCormick *et al.*, 1997; Piotter *et al.*, 1997). Compact discs (CD) are well-known examples for injection molded microstructured products. However, special care should be taken for microstructures with high aspect ratios. An elevated temperature is reached before injection molding. For amorphous thermoplastics (e.g. PMMA, PC), these temperatures are above their T_g. For semi-crystalline thermoplastics (e.g. POM, PA), crystallite melting points are often chosen. Prior to demolding, both the polymer substrate and the molding tools with mold inserts have to be cooled to a demolding temperature determined by the material and the specific patterned microstructures. Other peripheral

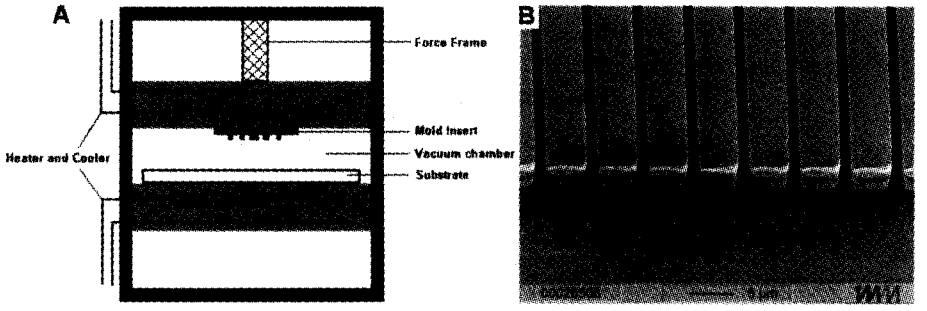


Fig. 4. (A) Schematic diagram for a hot embossing machine. (B) Microchannel structures created on PMMA by hot embossing. Reprinted with permission from Fan and Harrison (1994).

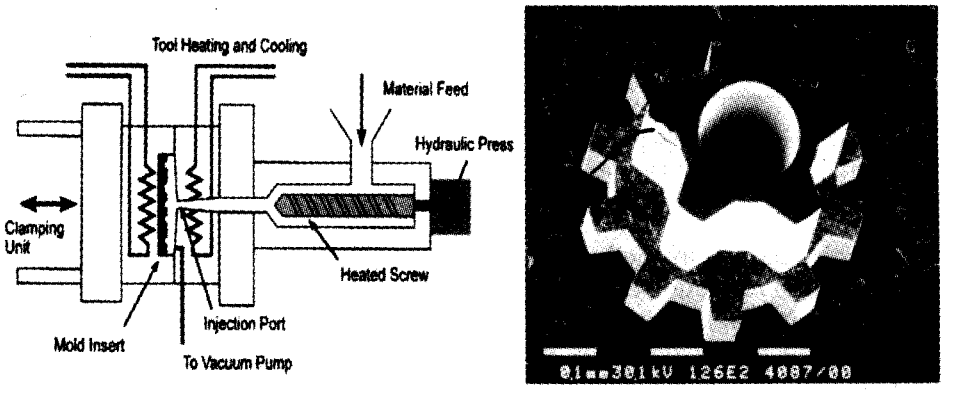
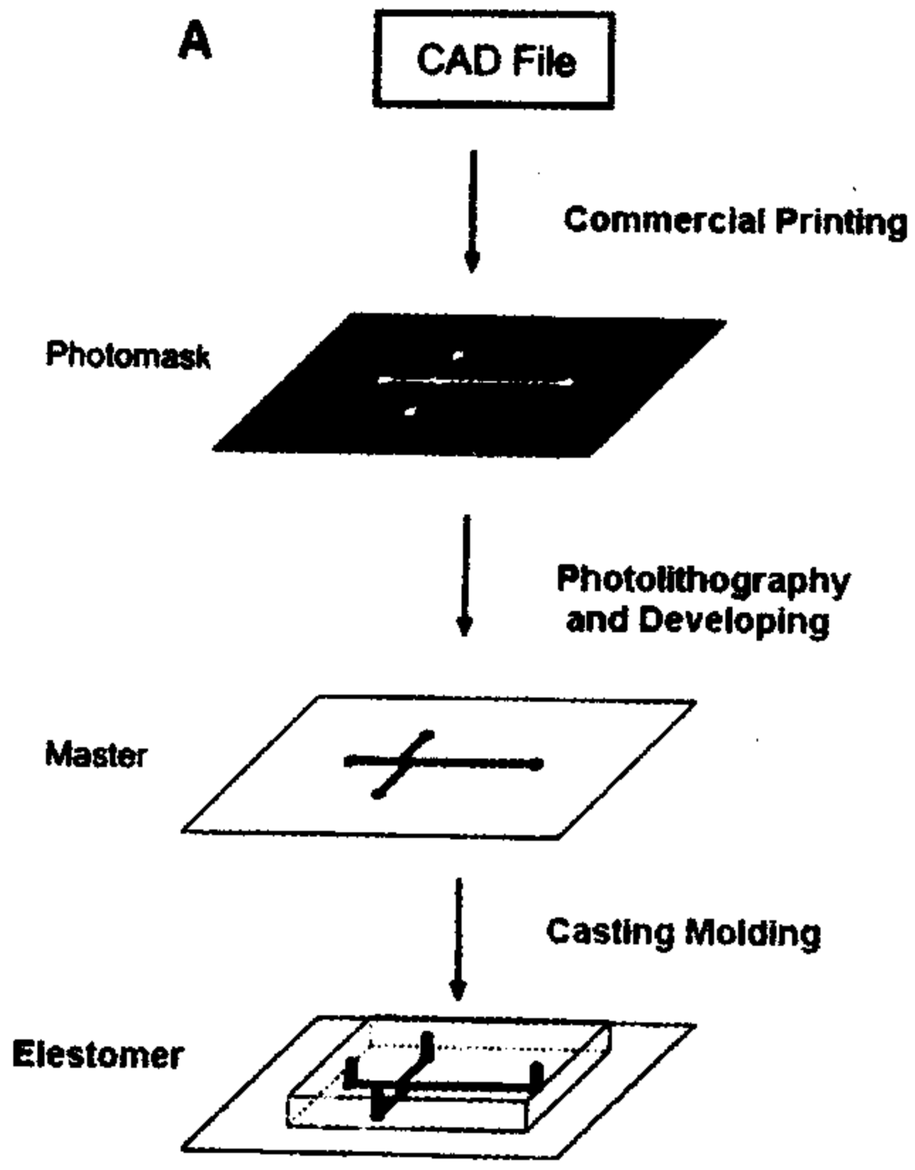


Fig. 5. (A) Schematic diagram for an injection molding machine, reprinted from Becker and Gärtner (2000) with permission. (B) Microgearwheel structures manufactured by injection molding, reprinted from Piotter *et al.* (1997) with permission.

equipment including a special vacuum unit is also needed for evacuation of the mold cavity in the molding tool, as well as a temperature control unit. Diagrams of an injection molding machine and a view of microchannels fabricated using injection molding are shown in Figure 5A and B.

For silicone-based elastomers, a casting process is the easiest and most widely used way of fast fabricating microfluidic devices (Qin *et al.*, 1998; Effenhauser *et al.*, 1997). This type of fabrication is extremely suitable for PDMS elastomers. During the casting process, a mixture of elastomer prepolymer and curing agent are cast against a master with a negative relief, degassed under vacuum and then heated to initiate the polymerization (typically 60~90°C and lasting 1~2 h for PDMS). After the elastomer is cured, the replica is then peeled from the master, and sealed with another planar sheet, forming an enclosed microchip.

Two classes of technologies, soft lithography and rapid prototyping are the most common methods employed for creating a master. Soft lithography is a suite of non-photolithographic methods for replicating patterns. An elastomeric structure with patterns embedded as a bas-relief on the surface acts as the pattern transfer agent.



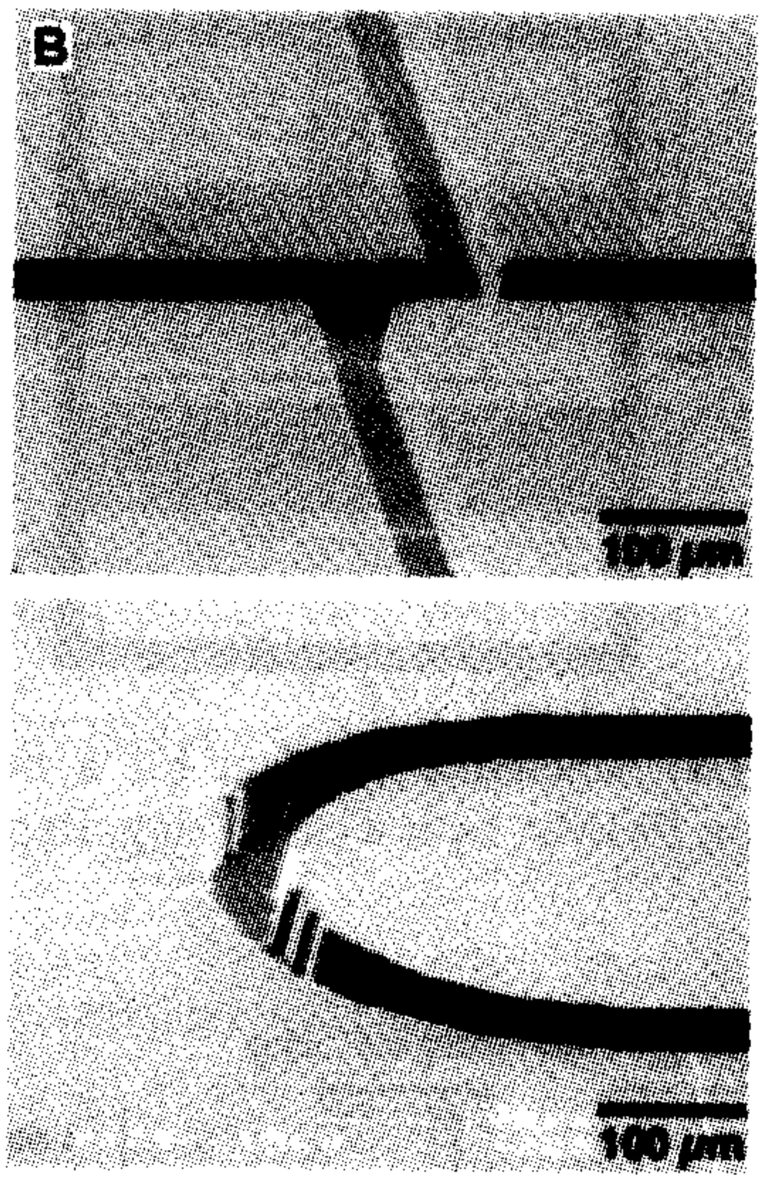


Fig. 6. (A) Scheme for rapid prototyping and casting molding. (B) Microstructure formed on PDMS fabricated by casting molding. Reprinted from Duffy *et al.* (1998a, 1998c) with permission.

The soft lithography methods do not require routine access to a clean room for replication resolution down to < 1 μm. Rapid creation of a master prototype (rapid prototyping) begins with creation of a design for a device in a CAD program. Then, the design is printed on to a transparency using a high-resolution image setter. This transparency later serves as the photomask in contact photolithography to produce a positive pattern of photoresist (e.g. Su-8). PDMS is cast against the master made of patterned photoresist, to form a negative relief (Figure 6A).

3.2.2.2. Direct technologies. Besides the above-mentioned techniques using replication processes to produce polymer devices from a single mold, there are several other techniques that allow individual micromachining of each single device. An obvious advantage of these techniques over replication techniques is that no master is needed, especially for manufacturing a single device or a few devices. A widely used technology for the fabrication of microfluidic devices is laser ablation (Roberts *et al.*, 1997; Pethig *et al.*, 1998). In this process, the energy of a laser pulse is used to break chemical bonds in a polymer molecule and to remove the decomposed fragments from the ablation region. With this technology, a wide range of polymeric materials including PMMA, PS, PC and others can be structured. An example of microstructure fabricated by laser ablation is shown in Figure 7A. Optical lithography refers to directly patterning a photosensitive polymer to form microchannels using lithography technologies. Thick photoresists such as Su-8 are applied for the fabrication materials using this technology (Guerin *et al.*, 1997). A view of microchannel fabricated by optical lithography is shown in Figure 7B.

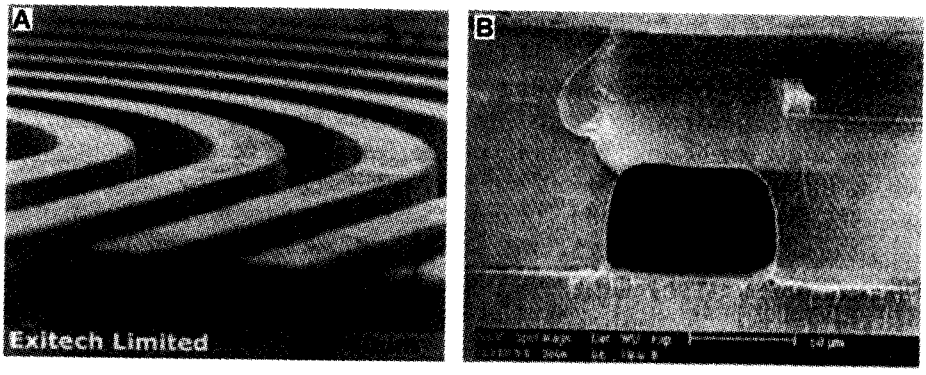


Fig. 7. (A) Microstructures fabricated using laser ablation. Reprinted from Becker and Gärtner (2000) with permission. (B) Microchannels formed in Su-8 photoresist fabricated using optical lithography. Reprinted from Fielden *et al.* (1998) with permission.

Stereolithography is another direct technology that allows actual three-dimensional microfabrication. In this method, a photocuring liquid polymer is exposed to a focused laser light. The polymer cures and forms a solid at the focal point. By moving focal point relative to the polymer, a three-dimensional structure can be constructed.

3.2.2.3. Sealing. Sealing is the last step for manufacturing microchips. For glass substrates, thermal diffusion bonding is the most often applied to enclose open channels (Woolley *et al.*, 1996). Other methods such as chemical-activated bonding and adhesive annealing are also used (Wang *et al.*, 1997). For polymeric substrates such as PMMA, PC, sealing with heat and pressure is normally utilized (Paulus *et al.*, 1998). Sealing with heat and pressure is different from hot embossing in that the temperature is below T_g of the polymer materials for sealing and above T_g for embossing. Care has to be taken not to damage the microstructures, thus this method is advisable mainly for designs with comparatively small structured areas, in comparison to the whole chip surfaces. Lamination is a well-known sealing process used for macroscale fabrication, and it has been transferred into microchip sealing (Roberts *et al.*, 1997). However, the adhesive sometimes tends to block the microchannels, and an inhomogeneous interface can be created, which may lead to band distortion during electrophoretic separation. A further two technologies, laser welding and ultrasonic welding have also been applied for sealing polymeric microchips which avoid the problems of adhesive.

4. DETECTION

The more fluidic functions are integrated on microchips, the more the necessity for high-performance detection is needed. Thus, the final success of a μ-TAS is increasingly determined by the ability of researchers and engineers to realize

detection methods that utilize the advantages of reduced diffusion lengths and confined geometries, while also solving the challenges imposed by such miniaturization. There are some requirements for microfluidic detection compared to those of conventional analytical systems. First of all, higher sensitivity is required, because of extremely small injection volume (typically in range of picoliters), as well as the minute detection cell size. Second, faster response times are also necessary, as the time for a sample band to pass the detector is much less than for conventional electrophoresis. Third, special structures are often required to match microchips with sophisticated detector designs. Fourth, the implementation of parallel detection is needed for high-throughput microchips. Last, portability and low cost are other two major requirements for successful microchip commercialization. Three major principles for analyte detection can be described: optical detection (laser-induced Fluorescence, absorbance detection, chemiluminescence detection and others), electrochemical detection (amperometric detection, conductivity detection and potentiometric detection) and mass detection (mass spectrometry). The following section is meant to give a brief introduction to the most widely utilized detection methods employed in chip-based CE.

4.1. Optical detection

4.1.1. Laser-induced fluorescence detection

Among the several above-mentioned detection methods, laser-induced fluorescence (LIF) is so far the most popular detection scheme and the first choice for detection of microchip separation because of its high sensitivity. Generally, detection limits down to 10^{-9}–10^{-12} mol/L can be obtained using LIF detection. With photon counting implemented in LIF detection, even single molecule detection can be achieved (Fister *et al.*, 1998). Both non-confocal and confocal detection systems are used for LIF detection of microchip separation (Jiang *et al.*, 2000). Confocal detection systems (Figure 8) have higher signal/noise (S/N) ratio than non-confocal detection systems, although a more complicated device setup is required. A typical confocal LIF detection involve the following steps: a coherent, collimated laser beam is reflected by a dichroic beam splitter into a high numerical aperture objective, which focuses the laser beam on inside the microchannel to excite fluorescently labeled analytes. The fluorescence emitted from the microchannel is collected by the same objective, and passes back through the dichroic beam splitter. In this same direction, the dichroic beam splitter reflects the laser while passes through the fluorescent analyte solution, which is then focused by another lens onto the entrance of a spatial filter (pinhole). The name "confocal" refers to the fact that this lens is confocal to the objective, that is to say, only light emitted from the focus of the objective can pass through the spatial filter, which will significantly increase the S/N ratio. Then, the fluorescence is directed to detectors, such as photomultiplier tubes (PMTs) or charge-coupled devices (CCDs), and an output signal from these devices will finally be recorded by a computer. There are some drawbacks of LIF detection however, such as the requirement of high-cost and cumbersome

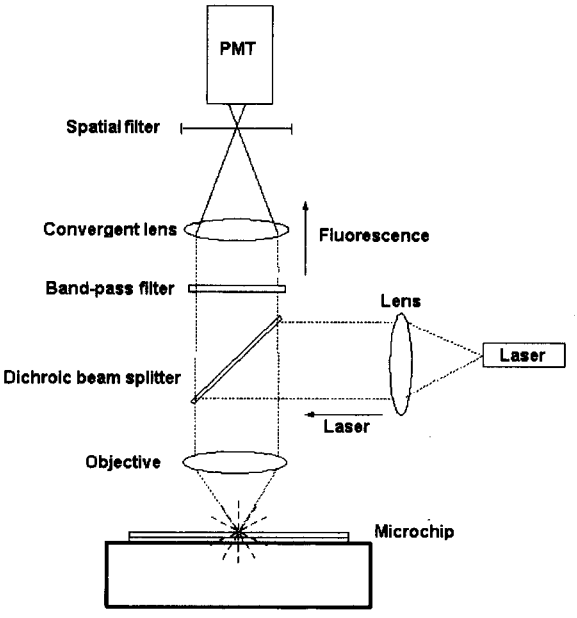


Fig. 8. Schematic view of LIF with confocal detection system.

laser instrument, optical alignment and the need for fluorescently labeled analyte. Therefore, great efforts have been made to ameliorate these costs and to develop various LIF detection systems with features such as device miniaturization and integration with the other microdevice components.

Integrated optics are an example of such a detection system, which aims at miniaturization and integration by utilizing light-emitting diodes (LED) in place of expensive and cumbersome external laser sources. As LEDs with higher output power and shorter wavelengths become available, more and more miniaturized detection systems have been developed using LEDs as light sources (Chabinyc *et al.*, 2001). Other groups have advanced further in developing integrated optics. For example, a blue GaN semiconductor LED, a CDs optical filter and a silicon photodiode were integrated into the same substrate by Chediak *et al.* (2004) to form an integrated optical system, with which the microfluidics component can be disposed after use, while the detection optics can be reused. Another example is from Webster's group (Webster *et al.*, 2001), who recently reported a combination of detector with optical interference filter constructed on the separation device, which has the advantage of the elimination of external optics and alignment procedures. Recently, another technique named liquid core waveguide has also been used in LIF detection. In liquid core waveguide mode, a special material (e.g., Teflon AF) functions as a liquid core waveguide. Excitation light impinges perpendicularly onto the capillary axis and passes through it, without being axially transmitted along the liquid core waveguide, while the fluorescence emitted by the dissolved analyte is transmitted along the waveguide and is then collected by an optical fiber positioned adjacent to the capillary outlet (Wang *et al.*, 2001).

4.1.2. Absorbance detection

Absorbance detection is quite popular in conventional CE, however it is not commonly used for chip-based detection because of the short optical path length in microchannels compromises the sensitivity. Nevertheless, various absorbance detectors have been developed for microchip format (Petsul *et al.*, 2001; Lu and Collins, 2001). In particular, absorbance detectors based on linear photodiode arrays have been developed to image the entire separation channel, which allows the direct visualization of the dynamics of entire IEF (Mao and Pawliszyn, 1999b) or electrophoretic separation processes (Nakanishi *et al.*, 2001). Other groups have devoted their efforts to increase the sensitivity of absorbance detection by increasing optical path length, by devices such as the use of multireflection cells (Salimi-Moosavi *et al.*, 2000) or by use of a thermal lens approach (Sato *et al.*, 1999).

4.1.3. Chemiluminescence detection

The term "chemiluminescence (CL)" was first coined by Eilhardt Weidemann in 1888, referring to the emission of light from a chemical reaction. Using CL as a detection method for chip-based CE has the following advantages. First, high detection sensitivity can be achieved comparable with that achieved with LIF detection. Second, a wide linear range of responding signals will be beneficial for quantitation of the analyte. Third, using CL detection eliminates the need for light sources, which are often expensive and cumbersome in LIF detection. Mangru and Harrison (1998) first demonstrated a CL system to monitor horseradish peroxidase (HRP) and fluorescein-conjugated HRP for microchip electrophoresis. Another CL detection system with still higher sensitivity was developed by Liu *et al.* (2003a) for microchip CE fabricated in PDMS.

Electrochemiluminescence (ECL) is also sometimes called electro-generated CL and it is a form of CL in which the light emitting chemiluminescent reaction is preceded by an electrochemical reaction. With ECL, the advantages of CL are retained, but the electrochemical reaction allows the time and position of the light emitting reaction to be controlled. Furthermore, an additionally beneficial aspect of ECL is that better detection limits may be achieved by rapid electrochemical recycling of reagents generating a rapid release of a chemiluminescent signal (Arora *et al.*, 2001).

4.2. Electrochemical detection

Although LIF detection is the most common detection scheme for microchip separation systems employed so far, its shortcomings are also obvious. Most compounds are not naturally fluorescent, and thus fluorescent labeling or a derivatization step is inevitable for LIF detection, which may artificially alter the separation properties of the analytes. Furthermore, the high cost and large size of the instrumental set up of LIF detection are sometimes incompatible with the concept of μ-TAS, especially with the applications when portability and disposability are necessary, such as point-of-care or *in-situ*

analysis. In contrast, electrochemical (EC) detection is an alternative detection mode that is ideally suited to miniaturization and thus suited to chip-based CE analysis. With photolithographic techniques that have already been used for constructing microchips, microelectrodes can be fabricated directly onto the microchip device, leading to a fully integrated system. Furthermore, miniaturization of electrodes does not compromise their sensitivity, unlike absorbance detection which is strictly governed by the length of optical path. Typical limits of detection for microchip EC detection are in the nanomolar range. Generally there are three modes of EC detection: amperometry, conductimetry and potentiometry. The following section will give a brief introduction to these modes of EC detection.

4.2.1. Amperometric detection

Amperometric detection is the most extensively reported EC detection method for chip-based CE, which may be considered in terms of electrolysis at a fixed point along a flowing stream. The stream here is a sequence of analyte zones separated with varying degrees of resolution. These zones pass into a detection cell, where a planar electrode is held at a fixed potential. If the potential is greater (more positive for oxidation or more negative for reduction) than that required for the electrolysis of the analyte, a measurable charge passes from electrode to analyte (or *vice versa*). The resulting current is directly proportional to the concentration of solute passing through the cell (Figure 9). The electrode may be thought of as a chemical reagent. The more positive its potential, the stronger an oxidizing agent it becomes; alternatively when the potential is made more negative, it becomes a stronger reducing agent. In either case, as the concentration of solute rises and falls in passing through the thin-layer cell, the electrolysis current proportionately follows these changes. This current, as a function of time, is

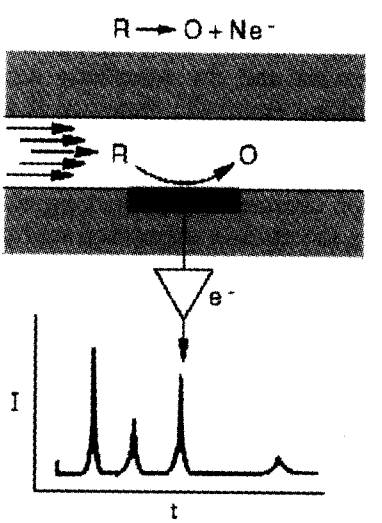


Fig. 9. Schematic diagram of amperometric detection. R and O refer to the reduced and oxidized states of the analyte, respectively.

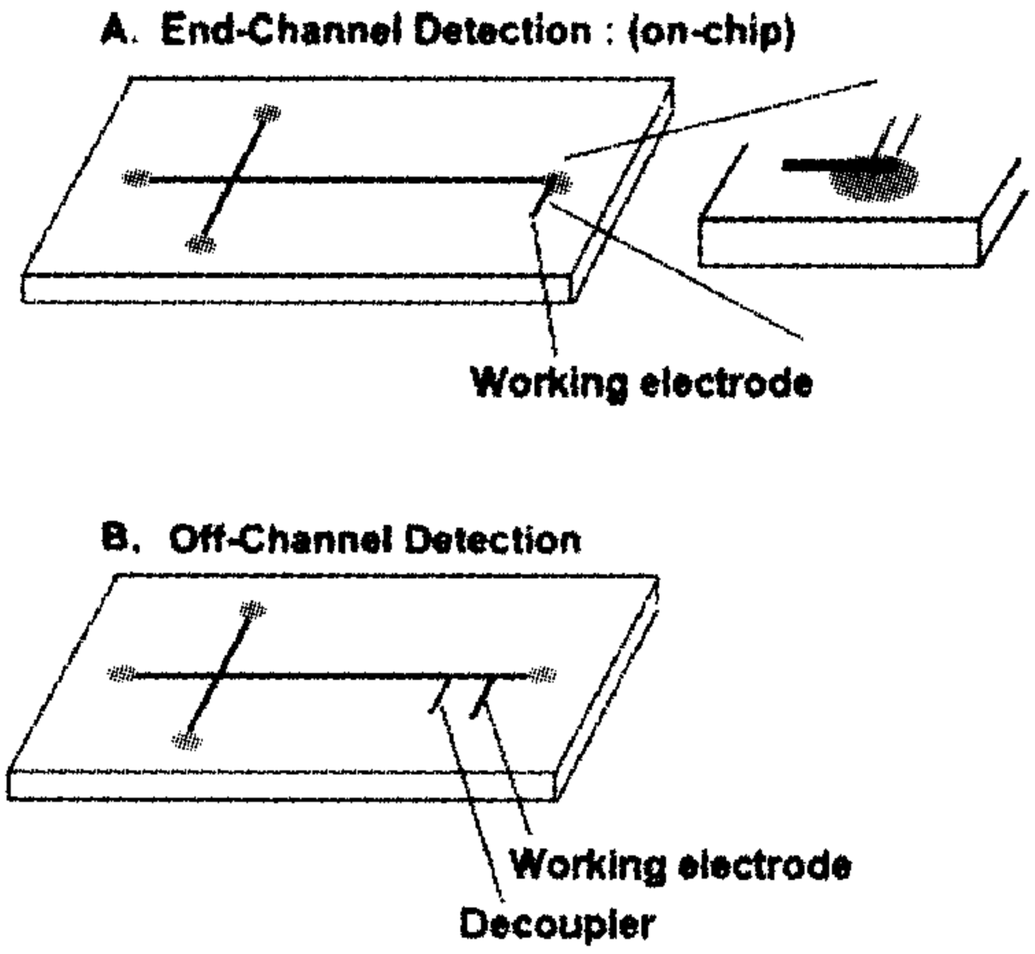


Fig. 10. Two configurations of working electrodes that isolate signal detectors from the high separation voltages.

amplified and sent to a recorder to yield a chromatogram. When utilizing amperometic detection in microchip electrophoresis, an issue of great importance should be addressed, that is to isolate the high separation voltage from the detector. Generally, two approaches have been developed for this purpose, termed end-channel detection and off-channel detection (Figure 10).

For end-channel detection mode, the working electrode is positioned tens of microns from the exit of the separation channel. This distance allows sufficient decoupling of the separation voltage from the detector. Amperometric detection in the end-channel mode was first reported for chip-based CE by Woolley *et al.* (1998). With their microchip electrophoresis system, neurotransmitters were successfully separated with good resolution, and attomole detection sensitivity was achieved. However, with their system, the separation voltage is grounded within the detection reservoir and the remaining separation field may cause potential shifts at the working electrode, therefore, it is necessary to perform a hydrodynamic voltammogram for each given analyte. Another drawback of end-channel detection is band broadening because the sample diffuses when passing from the exit of separation channel to the working electrode. Mathies and co-workers have developed a sheath-flow-supported scheme for end-channel detection to overcome part of this disadvantage (Ertl *et al.*, 2004). In their scheme, two sheath-flow channels were placed at a 30° angle relative to the separation channel, and were joined to it just before the end of the separation channel. A constant gravity-driven flow passed through the sheath-flow channels into the detection reservoir. These flows increased the velocity of analyte in the detection reservoir, which minimized the band broadening. Another way to eliminate band broadening is to employ off-channel amperometric detection. In this mode, the working electrode is placed directly within the separation channel, the analytes migrate over the electrode while still confined to the channel. However, a decoupler is usually needed for isolating the separation voltage from the amperometric detector (Osbourn and Lunte, 2003; Wu *et al.*, 2003; Lai *et al.*, 2004).

4.2.2. Conductimetric detection

Conductimetric detection is another EC detection method that is now gaining more popularity for use with chip-based CE. Conductimetry measures the differences in the conductivity of the bulk solution compared to that of the analyte zones, and can be considered a universal detection method because the analyte is detected without the need of fluorophore, chromophore or electroactive functional group detection. A typical conductimetry system employs two electrodes that are either in direct contact with the background electrolyte (contact mode) (Masár *et al.*, 2004) or are external and capacitively coupled to the solution (contactless mode) (Lichtenherg *et al.*, 2002; Laugere *et al.*, 2003). For measuring the conductivity, an alternating current (AC) potential is applied between the two electrodes, and when the ions of the background electrolyte are displaced by the analyte in the passing zone, a change in conductivity occurs, and the concentration of the analyte can then be correlated with the deviation of conductivity from the baseline. A major consideration for conductimetric detection is that the conductivity of the background solution must be different from that of the analyte. In addition, the electrolyte must be carefully considered as a highly conductive may also result in too high a background signal, which could possibly interfere with the detection of the analyte zones.

4.2.3. Potentiometric detection

Potentiometric detection is a technique used to measure the potential that arises upon a membrane between two solutions with different ionic activities, under conditions of on-current flow. When analyte solution flows through a semipermeable, ion-selective membrane of an ion-selective electrode, a potential difference between the activity of the external and that of the internal solutions of the electrode is created. This potential is measured against the fixed potential of a reference electrode and can be correlated logarithmically to the concentration of the analyte. However, potentiometry is a difficult technique to apply to the detection of separation-based systems, because it is difficult to create an ion-selective membrane, which must be semipermeable to multiple ionic species that are to be analyzed, but not highly permeable to the background buffer ions. Consequently, there are few papers that report use of potentiometric detection for microfluidic systems (Tantra and Manz, 2000; Ferrigno *et al.*, 2004).

4.3. Mass spectrometry

Mass spectrometry is a powerful alternative to optical and electrochemical detection techniques that can be used to identify unknown compounds molecularly, to quantify known compounds, and to elucidate the structure and chemical properties of molecules. Detection of compounds can be accomplished with very minute quantities (as little as 10^{-12} g, or 10^{-15} m for a compound of mass 1000 Da), which means that compounds can be identified at very low concentrations (one part in 10^{12}) in chemically complex mixtures. Thus,

examples of the application of mass spectrometric detection for low concentration and complex mixtures are increasing – is it increasingly being applied for the detection of modifications in the methylation state and other modifications of nucleic acids in biological systems (see this volume, Ehrich *et al.*, 2007). It is also very suitable for the study of proteomics with complex mixtures of proteins, or for small modifications in the abundance of modified species of proteins.

To date, most work on coupling mass spectrometry with microchip separation systems is focused on the design of the interfacing between the systems. Electrospray ionization is the most frequently used method of ionizing chemical or biological compounds at the interface between separation microsystems and mass spectrometer. An emitter is needed for this method, which delivers the sample liquid to be ionized by creating a strong electric field between the emitter and the mass spectrometer. The two most important properties of the emitter are – a low liquid flow rate that results in high ionization efficiency, and the sharpness of the emitter that leads to the creation of high electric fields. Many reports have described efficient sample introduction from microchip into mass spectrometer using electrospray ionization. For example, Schilling *et al.* (2004) have reported a new on-chip electrospray ionization nozzle that can be used as an interface for coupling microfluidic devices with mass spectrometric detection. The nozzle was micromilled in a polymer foil (PMMA, 750-μm thick) with three different inner nozzle diameters, and two different apex angles (Figure 11). The analysis of the tetrapeptide MRFA was readily achieved using this interface nozzle as stable electrospray conditions could be generated between the chip ionization system and the mass spectrometer.

Another example is provided by Tachibana *et al.* (2003), who have developed a robust and simple interface for microchip elecrophoresis-MS. A spray nozzle was connected to the exit of the separation channel of the microchip by use of a polyether ether ketone screw without glue, allowing easy replacement. Using this instrumental set up a few basic drugs were separated by microchip electrophoresis and the separation efficiency was improved by using high-viscosity separation matrix and a spray nozzle with small core size (20 μm).

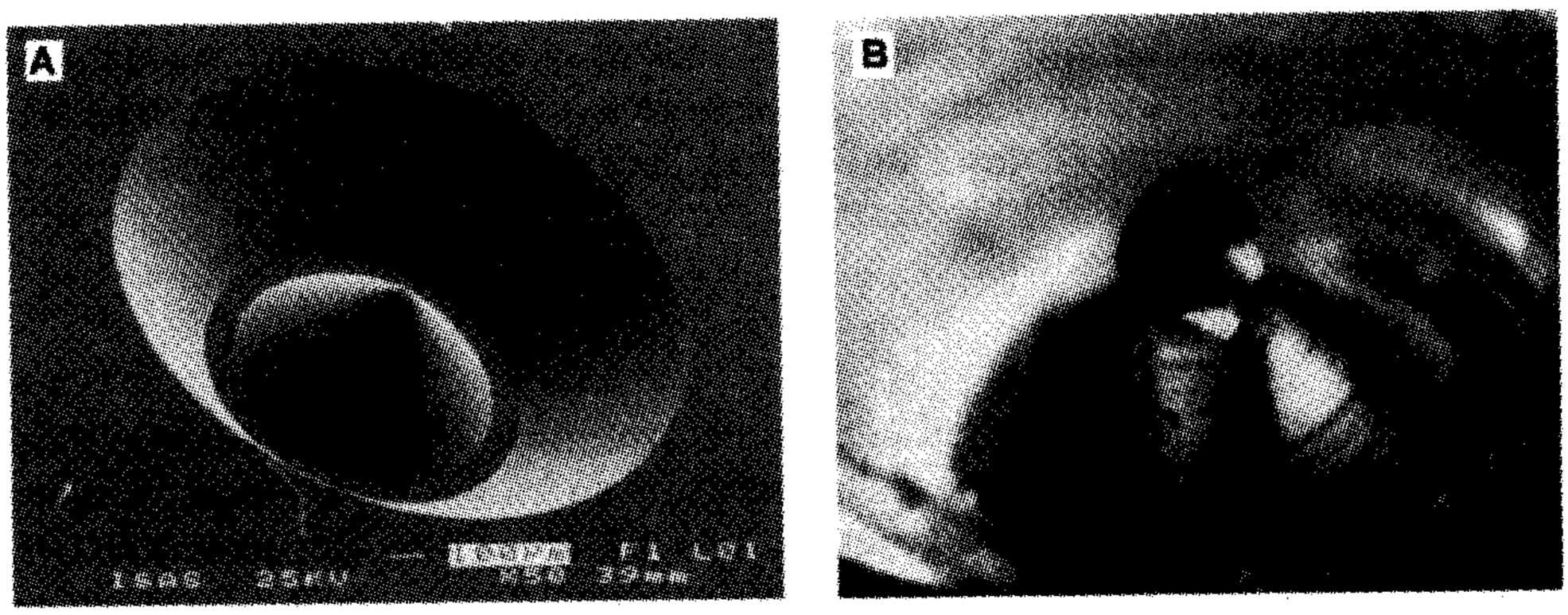


Fig. 11. (A) SEM picture of a micromachined ESI nozzle with an inner diameter of the tip hole of 40 μm and an apex angle of 90°. (B) Taylor cone at the nozzle tip with fluoropolymer coating. Reprinted from Schilling *et al.* (2004) with permission.

5. SURFACE MODIFICATION

It is well known that for conventional CE, the performance as well as the reproducibility of electrophoretic separations can considerably be improved by appropriate surface modification. Internal capillary coatings are typically applied to control the EOF and to suppress interactions between the analyte and the capillary wall, especially for separations involving macromolecules such as protein and DNA. Chip-based CE can be considered as a highly miniaturized version of CE, and with the reduction of the size of channels the properties of channel walls become more important than for conventional capillaries. Surface modification is a key factor for a successful demonstration of chip-based CE, as well as for other microfluidic device applications. A number of obvious advantages can result from choice of the appropriate surface modification, such as the change from hydrophobicity to hydrophilicity that enables use of polar liquids, and the generation or elimination of EOF effects that can facilitate fluid manipulation.

There are two major categories for surface modification of microchannel: dynamic coatings (physical-adsorbed coatings) and permanent coatings. Dissolved surface adsorptive compounds are usually used for dynamic coating of the channel. By rinsing the microchannel with a solution of the modifier prior to separation or by addition of the modifier to the electrolyte, microchannel can be dynamically coated with modifier, which then remains during electrophoresis. In permanent coatings, the coating materials are covalently bounded to functional groups on the microchannel surface and are thus immobilized and insoluble to the electrolyte. However, the achievement of internal surface modification for chip-based CE devices is particularly challenging, because chips are formed from diverse materials with various different inherent surface properties. Here, we focus only on the surface modification of the materials most widely used for microchips, such as glass, PMMA and PDMS.

5.1. Dynamic coating

Dynamic coating is the easiest way to achieve surface modification and is widely used for the control the EOF in microchip separations. Dynamic coating can be accomplished by adding selected surface-active compounds like polymers or surfactants to the running buffer and modifying the surface, and then applying a rinsing step to remove excess compound immediately prior to the separation step. Depending on the charge of the modifier compounds adsorbed to the microchannel walls, the EOF can be suppressed, enhanced or even reversed. For example, poly(dimethyl acrylamide) (PDMA), hydroxyethylcellulose (HEC), hydroxypropylmethylcellulose (HPMC) and hydroxypropylcellulose (HPC) are frequently used as dynamic coatings to diminish EOF and serve simultaneously as a sieving matrix for DNA fragments sizing (Bean and Lookhart, 1998; Albarghouthi *et al.*, 2003). To reverse the EOF, cationic detergents such as didodecyldimethylammonium bromide polycations, and cetyltrimethylammonium (CATB) can be employed (Landers *et al.*, 1992; Legaz and Pedrosa, 1996; Ding and Fritz, 1997; Melanson *et al.*, 2000).

5.1.1. Dynamic coating for glass/quartz substrates

For the dynamic coating of glass/quartz microchip substrates, the methods employed for conventional fused-silica capillary can be easily transferred to these devices. The dynamic coating of glass microchannels with PDMA is a well-known example, where 5% PDMA was used as a sieving matrix as well as dynamic wall coating in a 384-lane microchip for ultra high-throughput genetic analysis (Emrich *et al.*, 2002). In addition, various cellulose derivatives such as HEC, HPMC and HPC are also very popular buffer additives for producing dynamic coatings on glass chip surfaces used for DNA amplification and separation (Tian and Landers, 2002). Another non-conventional approach to dynamic coatings of glass substrates is the use of gold nanoparticles provided by Pumera *et al.* (2001). Here, the surface was first coated with a layer of poly(diallyldimethylammonium chloride) (PDADMAC) to support the adsorption of citrate-stabilized gold nanoparticles, which were subsequently collected on that surface. With the presence of gold nanoparticles on the channel surface, the resolution of aminophenol isomers was greatly increased due to selective interactions of the different solutes with this modified surface.

5.1.2. Dynamic coating for PMMA substrates

PMMA is an inexpensive polymer with good optical properties in visible light, which has been widely employed for fabricating microchips. However, its surface is rather hydrophobic and it exhibits moderate electro-osmotic mobility in aqueous solution, which is most likely due to non-esterified carboxyl groups. Several dynamic coating compounds have been tested for their ability to modify surface of PMMA microchips. For example, Xu *et al.* (2002) have reported separation of DNA using HPMC as a sieving buffer matrix on a PMMA microchip. To further improve analyte resolution, polyhydroxyl additives such as mannitol, glucose and glycerol have been employed as well. Another demonstration of dynamic coating is provided by Dang *et al.* (2003), who aimed at reducing analyte adsorption of microchannel walls by modifing PMMA surfaces using several low-molecular-weight compounds (amines, SDS, CTAB) and some hydrophilic neutral polymers (PEG, HEC, HPMC, MC). Oligosaccharide ladders could be successfully separated in surface-modified PMMA microchips.

5.1.3. Dynamic coating for PDMS substrates

PDMS is another popular material for making microchips because of its optical transparency and utility for fabrication. However the surface of PDMS is also hydrophobic similar to PMMA, making it difficult to fill the PDMS microchannels with aqueous electrolyte, and also because of its hydrophobicity, significant physical adsorption of proteins on PDMS surface has also been reported. Dou *et al.* (2002) utilized MES to modify surfaces of PDMS microchips, where the addition of 2-morpholinoethanesulfonic acid (MES) to the electrolyte buffer improved the separation efficiencies of analytes, such as

arginine, glucose and methionine-glycine. A dynamic coating composed from multiple layers of positively and negatively charged polymers was applied to PDMS chips by Liu *et al.* (2000). Polyelectrolyte multilayers were created by exposing the channel wall to alternating solutions of positively (polybrene) and negatively (dextransulfate) charged polyelectrolytes. The dynamic coating exhibited a stable and nearly pH-independent EOF in the range of 5–10.

5.2. Permanent coatings

Though much more laborious to apply than dynamic coating, permanent coating is considered as the most effective way to achieve surface modification because of its better stability and performance.

5.2.1. Permanent coating for glass/quartz substrates

The surface of glass substrate contains silanol groups, similar to fused silica, although at a lower density. Therefore, the first choice to permanently modify a glass surface is to undertake chemical reactions with its silanol groups. A permanent coating for glass/quartz microchips with linear polyacrylamide (LPA) proposed by Hjertén (1985) is now the most widely applied method. Briefly, microchannels are first flushed with NaOH, and then filled with γ-methacryloxypropyltrime-thoxysilane in diluted acetic acid and/or acetonitrile for 1 h. In a second step, an aqueous solution of acrylamide with ammonium persulfate (APS) and *N,N,N′,N′*-tetramethylenediamine (TEMED) is then pumped into the channels and polymerize at room temperature. Lastly, the channels are flushed with water and dried by vacuum. Glass microchips with this permanent coating have been widely used for genetic analysis.

5.2.2. Permanent coating for PMMA substrates

Permanent coating for PMMA substrates is employed to generate amine-terminated surfaces that could be utilized for immobilization of enzymes and dsDNA (Henry *et al.*, 2000). Further reaction of this surface with *n*-octadecane-1-isocyanate will generate an octadecane-chain-terminated surface, which could be applied to reversed-phase CEC separations of DNA ladder (Soper *et al.*, 2002). Another modification is to apply a pulsed UV excimer Laser (KrF, 248 nm) at sub-ablation fluency (Johnson *et al.*, 2001). Using low laser power, the surface chemistry of PMMA could be altered, producing carboxylic groups at the surface without changing the physical morphology.

5.2.3. Permanent coating for PDMS substrates

Treatments of the PDMS surface with oxygen plasma, UV light or corona discharge will replace methyl (Si-CH_3) groups with hydroxyl (Si-OH) groups, thus changing its hydrophobic surface properties from hydrophobicity to

hydrophilicity (Hillborg and Gedde, 1998; Efimenko *et al.*, 2002). However, oxidized PDMS surfaces are observed to exhibit dynamic surface properties due to recovery of hydrophobicity after oxidation, probably because of diffusion of low-molecular-mass PDMS chains in the polymer bulk onto the surface, or diffusion of the oxidized PDMS chains into the polymer bulk. Another approach namely radiation-induced graft polymerization can be employed in place of modification by exposure to the energy sources described above. If the polymer surface has no chemically reactive functional groups, irradiation would be needed to generate free radicals on the surface, which then act as sites for graft polymerization. Hu *et al.* (2002) demonstrated this radiation-induced graft polymerization process, by modifying the surface of PDMS with acrylic acid (AA), acrylamide (AM), dimethyl acrylamide (DMA), 2-hydroxyethylacrylate (HEA) and poly(ethylene glycol)mono methoxylacrylate (PEGA). The DMA and PEGA grafted microchannels were then selected for electrophoresis of two peptides, and both modified surfaces exhibited little adsorption of the peptides on the channel walls.

6. APPLICATIONS

As the technologies required for chip-based CE have matured, there have been a wide application of this platform for the analysis of small molecules such as amino acids (Jacobson *et al.*, 1998), dyes (Effenhauser *et al.*, 1993) and explosives (Wallenborg and Bailey, 2000), and also for macro molecules such as polysaccharides (Emrich *et al.*, 2002), proteins (Li *et al.*, 2004) and DNA (Dou *et al.*, 2002). It has also been readily integrated with other functional miniaturized instrument components to realize the concept of "lab-on-a-chip" (Manz *et al.*, 1990). However, here we only focus on the applications relevant to nucleic acid analysis by chip-based CE, and on integrated lab-on-a-chip devices (genetic micro total analysis systems, g-μTAS).

6.1. Nucleic acid analyses

Conventional CE offers many advantages over slab-gel electrophoresis in terms of higher resolution, shorter separation and lower sample consumption. The employment of high-throughput CAE sequencing methods also allowed the HGP to be successfully completed in advance of projected time. Chip-based CE is now widely considered as a viable alternative to conventional CE and will undoubtedly have a great impact on analytical science, especially for the analysis of nucleic acids. CGE with various different sieving matrices is always employed for the analysis of nucleic acids because of the nearly identical charge-to-mass ratios of nucleic acids regardless of chain length. Therefore, we will first give a brief introduction to sieving matrices utilized for nucleic acids separation. Then according to the resolution required, several specific applications including fragments sizing, genotyping and sequencing are discussed.

6.1.1. Sieving matrices

Although cross-linked LPA is widely used in slab-gel electrophoresis and was also employed in early CE development, there are several deleterious factors that hinder its use in chip-based CE systems. Several major factors are that both gel breakage and gas bubble formation can occur because of matrix shrinkage during *in situ* polymerization. In addition, the high field strengths and alkaline pH used for DNA separation exacerbate gel hydrolysis and degradation and result in short lifetimes and low reproducibility. Hence, for chip-based capillary electrophoretic analysis of nucleic acids, cross-linked gels have been replaced by non-cross-linked polymer solutions (Heiger *et al.*, 1990) and other novel types of matrix.

The main polymer solutions used as sieving matrix for nucleic acids analysis are listed in Table 2, along with their respective viscosities. These physical characteristics are a key factor in the efficiency of the matrix performance. Among the above-listed polymer solutions, the non-cross-linked, highly hydrophilic LPA solution provides the highest resolution, unsurpassed by other linear or branched polymers. Unfortunately, LPA has no self-coating ability, thus it must be used in pre-coated separation channels, whereas some of the other polymer solutions including PDMA, PVP, PEO and cellulose derivates (see Section 5), have self-coating properties and thus are also widely used for DNA separation.

Viscosity is an important factor in matrix loading due to the loading pressure limitation of the bonded microchip. For most glass chips, up to 200 psi pressure can be tolerated, while most plastic microchips can only tolerate 50 psi. However, it is generally true that higher resolution can be achieved using more concentrated polymer solution, which usually results in higher viscosity. To address this issue, several temperature-dependent viscosity-adjustable (thermo-responsive) polymer solutions have been developed for DNA separation (Albarghouthi *et al.*, 2001; Doherty *et al.*, 2004; Barron, 2004). These matrices contain two viscosity zones between loading and separating stages, allowing rapid loading with a lower viscosity and rehabilitating good separations with a higher viscosity. One type of thermo-responsive polymer solutions is the thermo-associating polymer solution, of which the viscosities increase with elevated temperature.

A good example is the low-molar-mass triblock copolymer of the poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) ($PEO_{99}PPO_{69}PEO_{99}$). At 5°C, a 25% w/v $PEO_{99}PPO_{69}PEO_{99}$ solution has a low viscosity of 50 cP, allowing for easy injection loading. When at 20°C, the viscosity increases sharply to 250 cP making it suitable for DNA fragments sizing. Another type of thermo-responsive polymer is the thermo-thinning polymer solution that has temperature-response characteristics opposite to that of thermo-associating polymers. For example, the copolymer of *N,N*-dimethylacrylamide (DMA) and *N,N*-diethylacrylamide (DEA) has a low viscosity of 10 cP at 70°C suitable for matrix loading, whereas at 40°C this matrix permits separation of DNA to be achieved at single-base resolution (Albarghouthi *et al.*, 2001).

Large DNA molecules are conventionally separated by pulsed-field gel electrophoresis (PFGE), which is extremely time consuming, with a typical running

Table 2. Polymer solutions used as sieving matrices for nucleic analysis

Polymer	Molar mass (kDa)	Concentration (wt%)	Buffer	Temperature (°C)	Viscosity (cP)
LPA	9000	2	50 mM Tris-TAPS, 2 mM EDTA, 7 M urea	25	27,400
PDMA	200	6.5	100 mM TAPS, 8 M urea, pH 8	30	1200
PDMA	98	6.5	100 mM TAPS, 8 M urea, pH 8	30	75
PEG	35	6	100 mM TAPS, 6.6 M urea		10,000
HEC	97	2	89 mM Tris-borate, 2 mM EDTA, 6 M urea, 10% formamide	25	5000
PEO	8000	1.5	89 mM Tris-borate, 2 mM EDTA	Ambient	1200
	600	1.4	3.5 M urea, pH 8.2		
PVP	1000	4.5	89 mM Tris-borate, 2 mM EDTA, pH 8.0	20	27
HPMC-4000		2	50 mM Tris-borate, 2.5 mM EDTA, pH 8.3		4390
HPMC-50		50	89 mM Tris-borate, 2 mM EDTA, pH 8.3, polyhydroxy	25	40
HPMC-5	5		100 mM Tris-borate, 2 mM EDTA, pH 8.0, mannitol	20	5.7
MC-4000	2		50 mM Tris-borate, 2.5 mM EDTA, pH 8.3	20	4390
MC-8000	2		50 mM Tris-borate, 2.5 mM EDTA, pH 8.3	20	7980
HPC-11000	2		50 mM Tris-borate, 2.5 mM EDTA, pH 8.3	20	11,000

Source: Reprinted with permission from Xu and Baba (2004).

time more than 10 h. A different idea for separating large DNA molecules involves fabricated nanostructures in the chip channel instead of using polymer solution-based sieving matrices. Entropic-based separation is a good example of this process, where separation is based on artificially fabricated nanofluidic channels with narrow constrictions that create mobility differences for separating long DNA molecules (> 5 kbp) without using polymer solutions or pulsed fields (Han and Craighead, 2000). The internal conformational entropy is one of the dominant properties of long flexible macromolecules, such as DNA, which is directly proportional to the molecular contour length. The entropic trapping

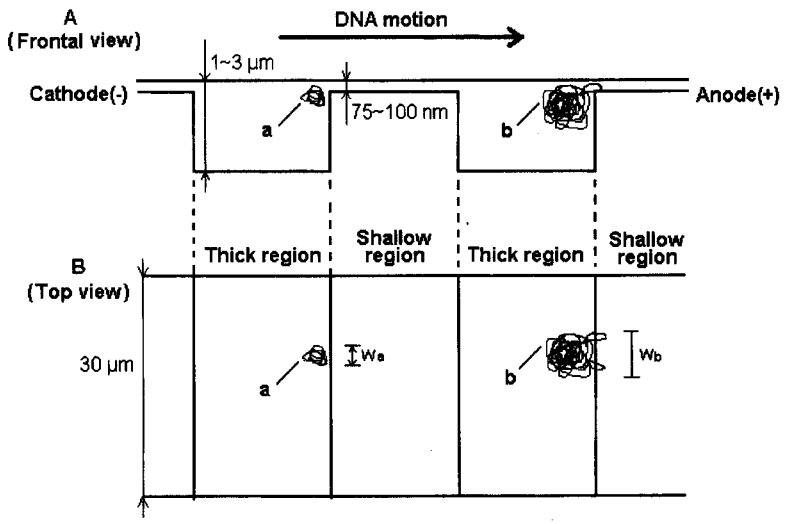


Fig. 12. Schematic view of entropic-based DNA fragment separation, where (A) illustrates the separation of smaller DNA molecules; and (B) illustrates the separation of larger DNA molecules. Reprinted from Han and Craighead (2000) with permission.

(ET) effect will occur when the radius of gyration (R_g) of DNA molecule is comparable to the mean pore size of separation gel. In entropic-based separations, DNA molecules are migrated by electrophoresis along a channel composed of a periodic array of alternate deep and shallow regions. In the deep regions the DNA molecules can form spherical equilibrium shapes, because the dimensions are much larger than the R_g of the DNA. In contrast, the height of shallow regions is much less than the R_g of DNA, thus the DNA becomes trapped at the entrance to the shallow regions and has to be deformed from its equilibrium shape to fit into the constriction, which can be used to determine the apparent mobility of DNA molecules. Longer DNA molecules will escape more easily from this ET than short molecules, because long molecules will have a larger contact area in the thin slit, resulting in a faster electrophoretic mobility. A schematic view of entropic-based separation is shown in Figure 12.

6.1.2. DNA fragment sizing

Fragments sizing requires the least resolution, and is thus the easiest achievable application of nucleic acid analysis. Various nucleic acids such as short oligonucleotides, polymerase chain reaction (PCR) products, restriction fragments and ribosomal RNA have been sized using chip-based CE, with some representative electropherograms shown in Figure 13.

6.1.3. Genotyping

Applications of chip-based CE for genotyping include analysis of genetic polymorphisms such as single-nucleotide polymorphism (SNP), single-stranded

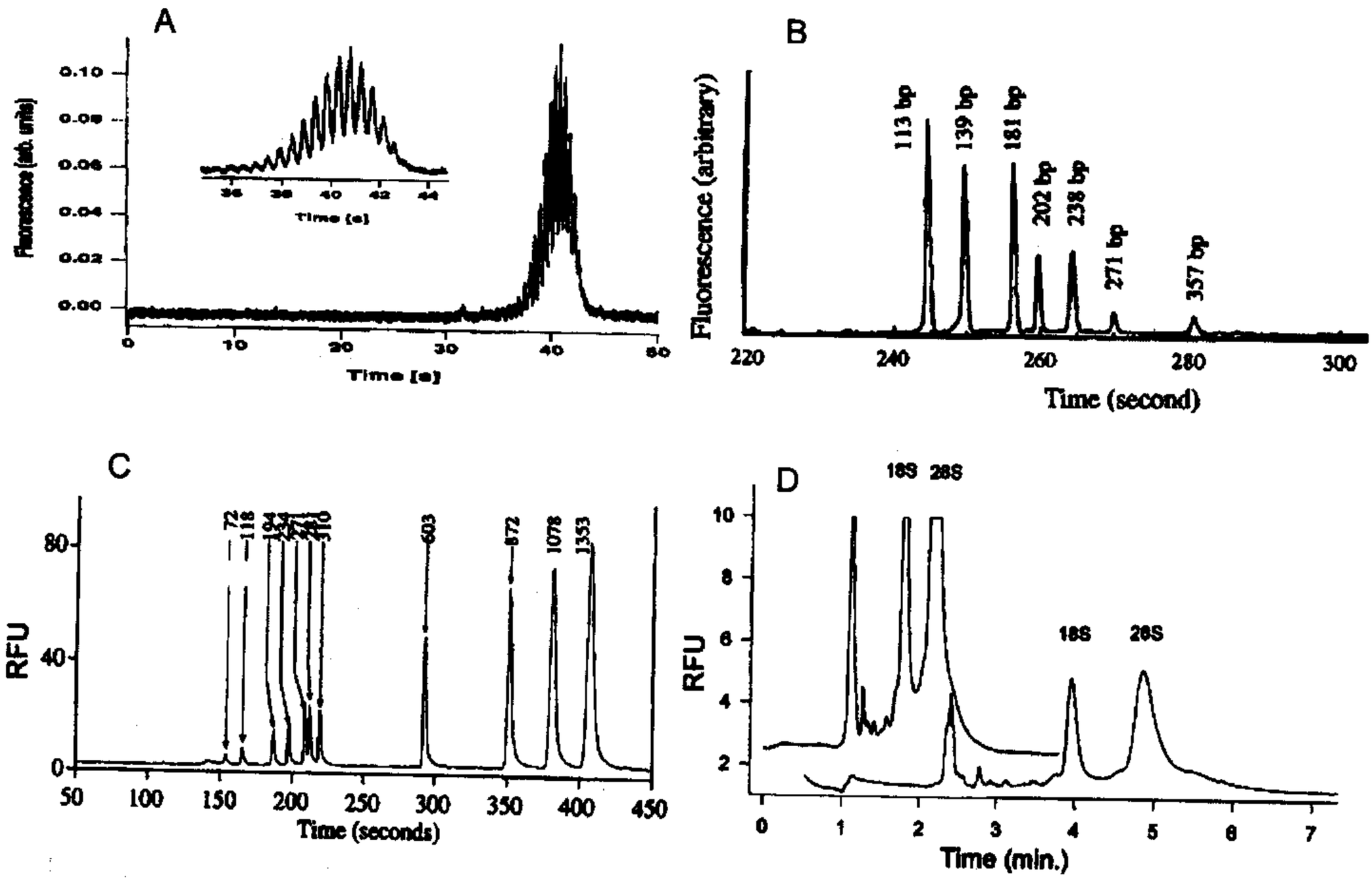


Fig. 13. Representative electropherograms of various nucleic acids fragments separated by chip-based CE. (A) Short oligonucleotide ladders, reprinted from Effenhauser and Mathies (1994) with permission; (B) PCR products, reprinted from Cheng *et al.* (1998c) with permission; (C) ΦX174 *Hae III* fragments, reprinted from Woolley and Mathies (1994), with permission; and (D) total RNA, reprinted from Ogura *et al.* (1998) with permission.

conformation polymorphism (SSCP), short-tandem repeat (STR) and so on. In these applications, efficient and sensitive separation of DNA molecules according to their size or conformation is required.

SNPs are the most abundant type of genetic variation in mammalian genomes. There are estimated to be about 3 million SNPs within an individual, and SNPs are widely used as markers for gene mapping and for genetic polymorphism analysis. SNP genotyping promises to reveal some of the genetic reasons why some people are more susceptible to diseases such as cancer or diabetes, and what predisposes others to suffer adverse reactions to drugs. As a result, SNP genotyping is a booming market with annual expenditure on SNP research predicted to grow from US$158 million in 2001 to more than $1.2 billion in 2005 (Melton, 2003). Most SNP scanning technologies utilize hybridization-based methods, however prior knowledge of the sequence of interest as well as the use of large amounts of synthetic DNA probes are often required (Park *et al.*, 2002; Wang *et al.*, 2003). Instead, high-throughput electrophoresis-based methods such as temperature gradient CE (TGCE) can also be applied for the discovery and mapping of SNPs. Liu *et al.* (2003b) have used chip-based TGCE for fast screening of SNPs, and some representative electropherograms are shown in Figure 14.

SSCP is an example of an alternative method for mutation detection based on electrophoretic DNA separations. SSCP analysis involves an electrophoretic separation of single-stranded nucleic acids based on differences in sequence.

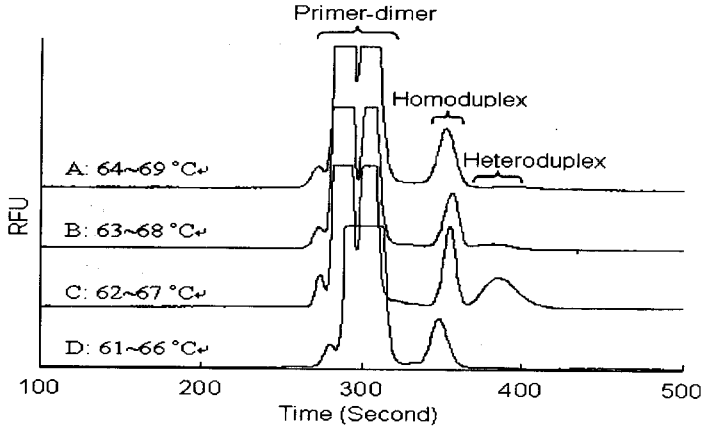


Fig. 14. Chip-based temperature gradient capillary electrophoretic separation of DNA fragments with two SNPs. Reprinted from Liu *et al.* (2003b) with permission.

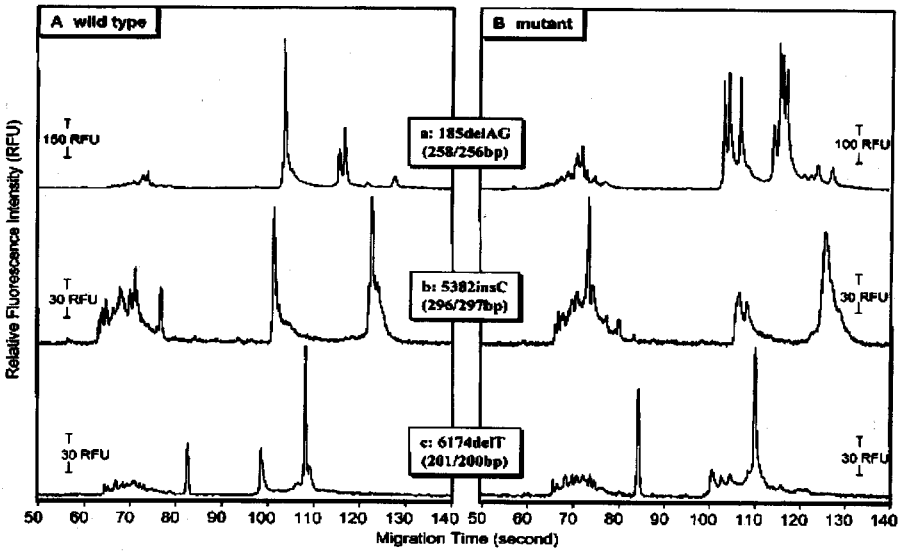


Fig. 15. Fast SSCP analysis of DNA fragments by chip-based CE. Reprinted from Tian *et al.* (2000) with permission.

Single base mutation may be detected because single base mutation may alter or disrupt the secondary conformation of the single-stranded nucleic acids sufficiently, resulting in an electrophoretic mobility shift. An example of mutation detection by SSCP analysis is provided by Tian Tian *et al.* (2000), who used both conventional and chip-based CE for detection of common mutations in BRCA1 and BRCA2, representative electropherograms are shown in Figure 15. Simple tandem repeats (STRs) are short stretches of repetitive DNA sequence that are distributed throughout the genome, typically, with each STR locus consisting of 7–20 repeats of specific 2- to 7-base sequences, resulting in another

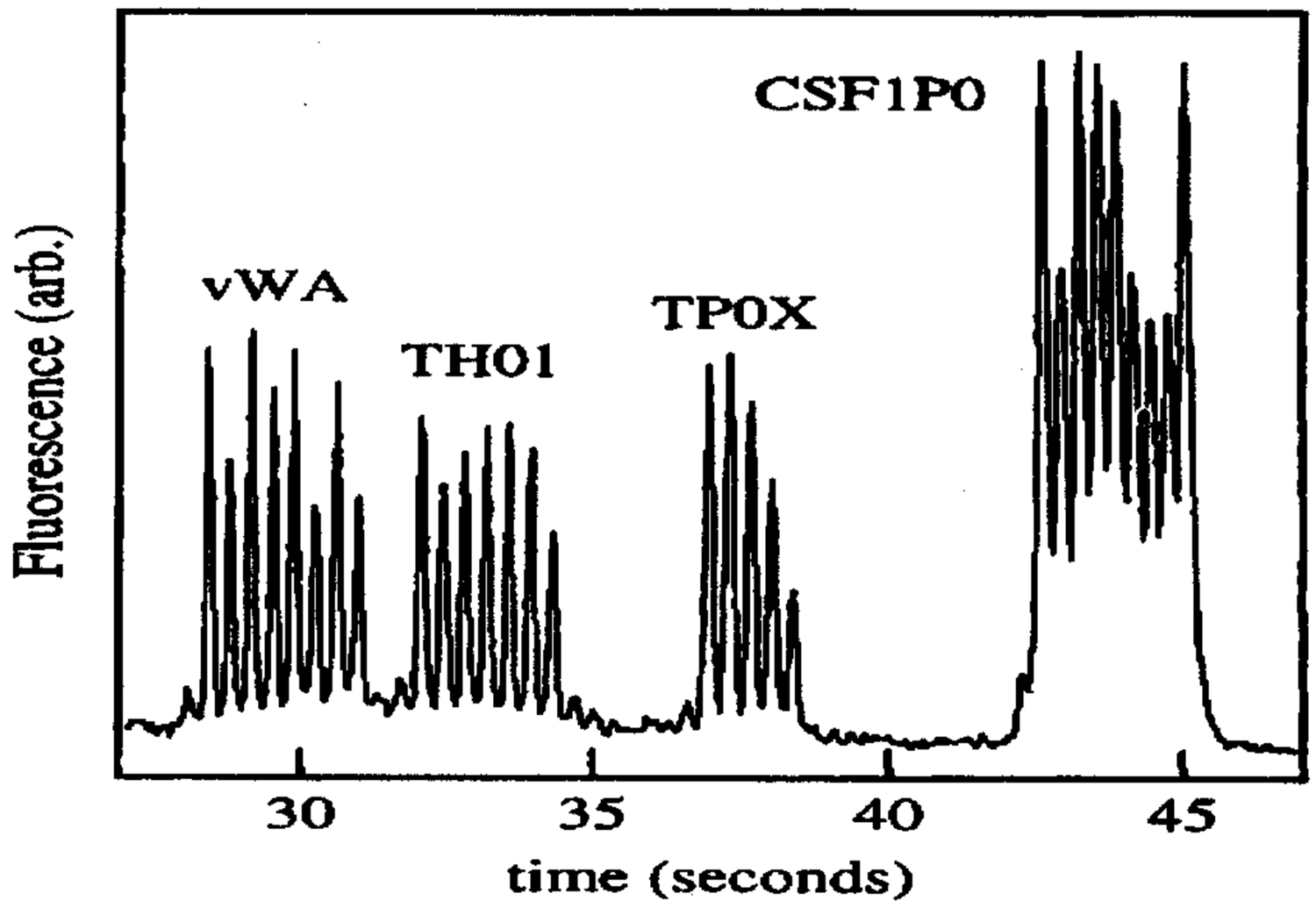


Fig. 16. Electropherogram of the four-locus CTTv allelic sizing standard. Reprinted from Schmalzing *et al.* (1997) with full permission.

common measurable genetic variation. STR analysis has developed as an important tool in forensic analysis, gene mapping and discovery, paternity testing and clinical diagnostics (Edwards *et al.*, 1991; Hammond *et al.*, 1994; Carey and Mitnik, 2002). Among various advances in STR typing technologies, the most popular is a PCR-based method with the product sizes analyzed by CE.

Resolution of 2–3 bp over several hundred bp is required to achieve unambiguous resolution of the different STR alleles. Schmalzing *et al.* (1997) demonstrated single-channel microchip STR analysis using a 2.6 cm separation length and a replaceable LPA matrix to analyze both fluorescently labeled CTTv PCR samples and STRs (shown in Figure 16).

7. DNA SEQUENCING

DNA sequencing has become an area of intense interest as one of the most important scientific accomplishments in human history is the completion of the human genome sequence in 2003 by an international collaborative Human Genome Program, coinciding with the 50th anniversary of the discovery of the DNA double-helix structure. The present state-of-the-art method for DNA sequencing still relies on an advanced form of the Nobel Prize-winning Sanger dideoxy chain termination reaction (Sanger *et al.*, 1977). Briefly, the genome is fragmented into small pieces, and the Sanger reaction (a controlled interruption of the enzymatic replication of an ssDNA template by a DNA polymerase) is used to produce a ladder of template-complementary DNA fragments that differ in length by one base and bear unique fluorescent labels, according to their terminal base. Electrophoretic separation of DNA fragments with single-base resolution is then applied, detecting the base-specific labels and reading the base sequence of each fragment into their original order. Until 1999, the majority of all DNA sequence had been generated by a manual slab-gel

electrophoresis process. Subsequently, CE, because of its speed and ease of process automation became a realistic option for increasing the throughput of any sequencing project. It is because of this successful transfer from slab-gel-based to capillary-array-based instrumentation that such a tremendous progress in data accumulation by HGP was achieved (Collins *et al.*, 2003). In the February 15, 2001 issue of *Nature*, the International HGP consortium published the draft sequence and initial analysis of the human genome, only several years after this transfer of genomic sequencing to array-CE.

Initially cross-linked polyacrylamide was employed in CGE, but this separation medium created practical difficulties for the preparation, operation and indeed the shelf life of capillaries filled with such materials. Karger and colleagues (Heiger *et al.*, 1990) soon developed a replacement for it with linear LPA in 1990. Among many materials employed for DNA sequencing, LPA shows the best performance: a 2% w/w solution of high molecular weight (M_r) LPA-producing single base-length fragment separation for more than 1000 bases in 80 min, which permits a read accuracy of approximately 97% was reported by Karger's lab in 1996 (Carrilho *et al.*, 1996). The same group continued their efforts of improving the performance of LPA by additional fine-tuning of the LPA composition, as well as optimization of electric field strength, run temperature and dye chemistry. The sequencing of 1000 bases in less than 1 h with a base calling accuracy of 98–99% was achieved using the optimized 2.5% LPA matrix, consisting of a mixture of 2% w/w high M_r LPA and 0.5% w/w low M_r LPA operated at 60°C and 200 V/cm (Salas-Solano *et al.*, 1998).

LPA is also not the sole choice of sieving matrix for DNA sequencing. Yeung and colleagues (Fung *et al.*, 1998) introduced the use of replaceable linear poly(ethylene oxide) (PEO) for DNA analysis, and Kim and Yeung (1997) reported the separation of a single-color sequencing ladder up to 1000 bases (resolution of raw data = 0.5 at base number 966) in a mixture of 1.5% high M_r PEO (8 MDa) and 1.4% low M_r PEO (600 kDa) at 75 V/cm over a separation capillary distance of 70 cm.Yeung's group also explored the use of polyvinyl pyrrolidone (PVP) for DNA sequencing, which is a self-coating material. Single-color sequencing with 7% commercially available PVP (1 MDa) showed reasonable separation up to 350 bases. Separation could be extended to 530 bases when a 5% solution of high M_r PVP extracted from the 1 M Da material was used. The separation was performed at 150 V/cm and room temperature with an effective column length of 50 cm, and the 530 bases eluted in 83 min (Gao and Yeung, 1998). Poly(dimethylacrylamide) (PDMA) is another self-coating material for DNA sequencing. In four-color sequencing runs, approximately 600 bases could be analyzed with a final resolution of 0.59 and a total run time of 125 min.The run conditions were 6.5% w/v PDMA at 160 V/cm and 42°C with an effective separation distance of 40 cm (Madabhushi, 1998).

Although CE with polymer solutions provides both better read-lengths and faster analysis speed (more than 1000 bases in less than 1 h) compared to that of slab-gel electrophoresis (routinely 600–700 bases requiring up to 10 h), unfortunately, this was not sufficient to compete with the throughput capable on the parallel lane slab-gel instruments. To turn it into parallel equipment with the required DNA sequencing throughput, CAE was introduced in 1992, combining

the high efficiency of conventional CE and the parallel feature of slab-gel electrophoresis (Huang *et al.*, 1992). Several different commercial capillary array sequencers are now available and this powerful instrument soon became the major workhorse for *de novo* genomic DNA sequencing. The two most powerful commercially available capillary array sequencers of that era were the ABI PRISM 3700 DNA Sequencer from PE Biosystems (Applied Biosystems) and the MegaBACE 1000 from Molecular Dynamics (GE Healthcare, Amersham-Pharmacia Biotech), both of which could analyze 96 samples per run. The MegaBACE 1000 DNA sequencer was based on the system developed by Mathies and colleagues (Huang *et al.*, 1992). It had automated sample and separation-matrix loading with a total turnaround time per sequencing run of less than 2 h between subsequent injections. This sequencer had confocal detection consisting of a microscope objective for focusing the laser light inside the capillaries and at the same time for collecting the emitted light from excited fluorophore tags at the center of the fluid column. A scanning system was used to collect the signal from all capillaries. With the MegaBACE 1000 Long Read Matrix, which contains LPA, the average read length exceeds 600 bases. The ABI PRISM 3700 DNA Sequencer from PE Biosystems was based on the developments of Kambara and Takahashi (1993) Dovichi (1997) employed post-column detection with liquid sheath flow. The capillary bundle was aligned inside a quartz cuvette. A buffer solution was pumped through the cell, along the dead space between the capillaries and the walls of the cuvette, where the liquid sheath flowing on the outside drags down the DNA zones eluting from each of the columns, tapering them to a small diameter without mixing. A laser beam crosses all flow streams and excites the fluorescent molecules. Light collection is made at 90° from the laser plane, and fluorescent light is imaged onto a cooled CCD camera for detection. Later iterations of these two systems employed 384 capillaries, yet despite the increased throughput these Sanger sequencing machines are now seen as limited in the present era of mammalian genome sequencing (with multigigabase data generation) and comparative genomics. Thus, the limitations of both throughput and sensitivity of these sequencing instruments have become critical as this technology was initially developed some 15 yr previously and require significant amounts of costly consumables (separation matrix, fluorescent sequencing chemicals, etc). Many researchers and companies are making concerted efforts to develop more powerful sequencers. These efforts include the development of integrated microchip bioprocessors that continue to use nanolitre amounts of Sanger sequencing chemistries (see Sections 7.1 and 7.3). Several of these other technology developments for DNA sequencing are also detailed in this volume, including, nanopore systems for single molecule analysis (Lee and Meller, 2007), and integrated solid-phase sequencing by synthesis systems that avoid use of electrophoretic fragment analysis characteristic of Sanger sequencing (Margulies *et al.*, 2007; Edwards *et al.*, 2007; Hebert and Braslavsky, 2007).

Another major development that led to integrated systems in which all steps of DNA sequencing analysis could be performed automatically was devised by Tan and Yeung (1997). They developed an integrated sequencer that included thermal cycling, product purification, in-line loading and CAE separation.

A dye-labeled terminator cycle-sequencing reaction is performed in a fused-silica capillary. The sequencing ladder was directly injected into a size-exclusion chromatography column at 95°C for purification. Online injection into the capillary was accomplished at a junction. The system was subsequently improved to process eight samples simultaneously (Tan and Yeung, 1998). The raw data allowed base calling up to 460 bases with an accuracy of 98%, and the design was scalable to 96 capillary arrays. Another general problem with most capillary array instruments was the need to extend read length, as it is generally difficult to achieve sequencing to at least >800 bases with >99% accuracy. Endo *et al.* (1999) proposed electric field strength gradients, with a typical duty cycle of an initial voltage ramp (up to 220 V/cm), for accelerating short fragments, followed by a plateau, a voltage decrement and finally a constant, lower voltage of 90–130 V/cm for separation of longer DNA fragments. These electric field strength gradients, coupled to column temperatures of 60°C, allow extension of the reading length up to 800 bases and similar measures are employed in current commercial 384-lane capillary instruments.

7.1. MicroChip DNA sequencing

However, the goals for the future of genomic research are not satisfied with currently developed CAE technology, and new advances in ultrahigh-throughput sequencing technologies will continue to be demanded for a significant period of time. The re-sequencing of a spectrum human genomes from different ethnic groups is only one project among the almost 100 genomes of other model and domestic organisms currently being sequenced, and a major concern is that the high cost of Sanger sequencing has become a significant obstacle to the continued genome sequencing of other mammals and to future progress in genomic science. To stimulate further technology development, an ambitious goal was set by the National Human Genome Research Institute (NHGRI) to develop tools capable of sequencing a human individual's genome for under $1000, in anticipation of a future genomic medicine. Here, chip-based CE appears promising as one emerging technology potentially capable of interim cost performance, while delivering long sequence reads typical of CE. Sequencing on chip-based CE offers many advantages over conventional CAE methods. A typical run time on microchip is in ranges of seconds to minutes, an order of magnitude less than that required for conventional methods. Furthermore, using lithography and MEMS technologies, microchips with high lane density and compactness can be easily fabricated, allowing massive parallel analysis and increasing throughput yet further. Another advantage provided by microchip format is low sample consumption that reduces the cost, typically picoliters of sample consumed for each run, compared with nanoliters required for conventional CE and microliters for slab-gel electrophoresis.

DNA sequencing with microchips was first demonstrated in 1995 by Mathies's group (Woolley and Mathies, 1995), who achieved a single-base resolution read length of 150–200 bases in 10–15 min with a separation channel distance of 3.5 cm using glass microchip with a denaturing LPA sieving matrix. Later efforts

have been made to improve the read length, as well as the throughput (multichannel microchip). Schmalzing *et al.* (1998, 1999) evaluated the relationship of sequencing read length with separation length and applied voltages in microdevices. Employing a single lane microchip, they separated up to 400 bases in 14 min at 200 V/cm by using a 4% solution of LPA. In a second approach, Liu *et al.* (1999) after optimizing some experimental parameters (extension of the separation channel length to 7 cm, use of optimized LPA, increase of the electrophoresis channel depth to 50 μm, and the use of low-fluorescence background borofloat glass wafers) reported sequencing read lengths of over 500 bases in 20 min with a accuracy of 99.4%. In a separate research development, the optimization of DNA sequencing was performed on a chip device containing a 150 μm twin-T injector and an 11.5-cm long separation channel (Salas-Solano *et al.*, 2000). Using a separation matrix composed of 3% w/w 10 MDa plus 1% w/w 50 kDa LPA, an elevated temperature (50°C) and 200 V/cm field, high-speed DNA sequencing of 580 bases was achieved in 18 min with a base-calling accuracy of 98.5%. This read length extended to 640 bases at 98.5% accuracy by reducing the electric field strength to 125 V/cm and with an increased analysis time of 30 min. Backhouse *et al.* (2000) reported an increased sequencing read length of 640 bases with 98% accuracy by using a 50 cm long microchannel at room temperature and 200 V/cm, utilizing POP-6™ as sieving matrix, showing that the performance of the microchip was identical to a fused-silica capillary with similar cross-sectional area.

Although problems still remain in the performance of microfabricated sequencers, significant advances have been made since Heller quoted that

> "for high-resolution applications such as DNA sequencing, the use of miniaturized separation devices in matrix-based electrophoresis remains an illusion". (Heller, 2000)

This was because base-call 600 or 800 bases, a separation length of at least a minimum of 14 cm, or as much as 30 cm, is usually required, a path length thought incompatible with microchips. An initial effort toward high-throughput DNA sequencing with long path multichannel microdevices was made by Ehrlich and colleagues (Koutny *et al.*, 2000), which with some 32 identical, separate 40-cm long channels, each with a 15 μm twin-T injector were fabricated on a 50-cm long and 25-cm wide glass chip, was hardly a microdevice. The separation achieved however was excellent, with average read lengths of up to 800 bases at an accuracy of 98%, and with a separation time of 80 min.

Mathies' group took up this challenge and devised long functional channel lengths within a small planar area by using tapered turns to prevent fragment separation distortions due to different path lengths across a turning channel (Paegel *et al.*, 2002, 2003). Signal detection in their microfabricated 96-lane radical CAE processor was by a 4-color rotary confocal fluorescence scanner, and the tapered turns extended the separation path length to 15.9 cm on a compact 150-mm diameter wafer. They obtained an average read length of 430 bases with a quality of PHRED >20. The sequencing output of one lane that exhibits average performance of this device is shown in Figure 17.

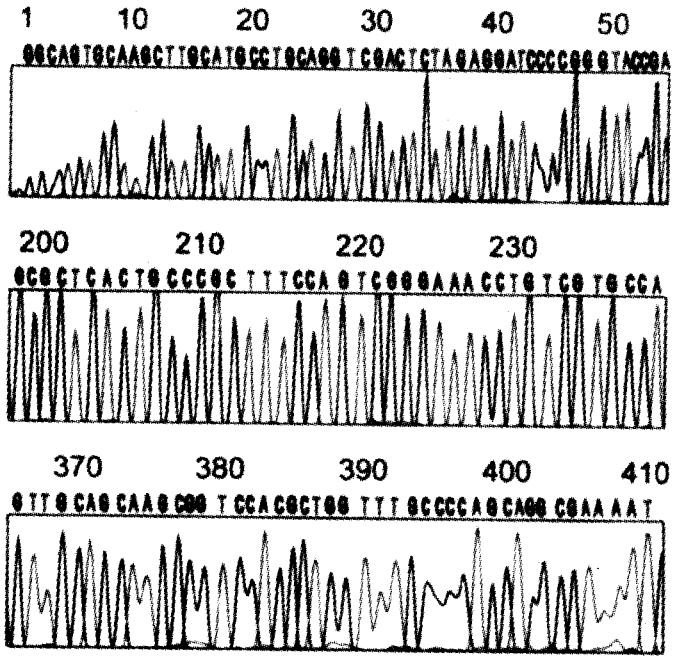


Fig. 17. The processed sequencing output display from a lane that exhibits the average performance of the system. The three panels display the typical quality of data obtained at the start, middle and the end of the run. Reprinted from Paegel *et al.* (2002) with permission.

7.2. Total genetic analysis systems

An intense area of development involves the creation of total genetic analysis systems (G-μTAS), formed by the integration of chip-based CE with other functional microfabricated components for processes such as sample preparation and other enzymatic bioreactions (PCR, restriction, labeling, etc.), and also with assay detection (such as fluorescence or CL) and potentially with other detection methods (such as microarray hybridization technologies). There are several advantages of the integration of such microanalysis systems. First, scaling down can increase the sensitivity. Suppose a single gene of interest is to be analyzed within the volume of a typical mammalian cell, where the concentration is of the order of 10^{-12} M. However, if it is diluted into a more conventional analysis volume of 10 μL, the concentration would drop to $<10^{-18}$ M, which makes it impossible to detect even with the most sensitive systems. Second, integration can eliminate external contaminations since the analytes need no longer be open to the environment. Third, reduction to a microscale makes it possible for batch fabrication to process many systems in parallel, as well as to fabricate many parallel systems on a single wafer, which can serve a unique role in clinical and research settings, since parallelism using conventional technologies is often prohibitively difficult. Finally, automation, portability and disposability can also be realized in an integrated microsystem, which are all key

factors for successful commercialization and industrialization. Typically, conventional genetic analysis consists of three separate tasks: sample preparation, bioreactions and detection. For sample preparation, several time-consuming steps are involved, including cell culturing, nucleic acids extracting and purification. Then, bioreactions such as PCR amplification and enzyme digestion are carried out. Finally, samples of interest are detected via electrophoresis (Harrison *et al.*, 1993) or hybridization. Prior to realizing a total nucleic acids system, successful transfer of the above-mentioned tasks onto microchip format must be carried out separately. Then, these functional parts must be integrated by sophisticated fluid manipulation using microfabricated pumps and valves.

7.2.1. Sample preparation on microchips

Sample preparation is essential for all bioanalysis and is a step with the most diverse procedures. Therefore, various techniques have been developed for different purposes, such as cell trapping, or selection from an undesirable context via dielectrophoresis (DEP) or filtration, then cell lysis using ultrasonics or electric fields, and finally sample concentration or dilution. Some different representative techniques that have been enabled on microchip devices are illustrated in Figure 18.

7.2.2. Bioreactions on microchips

PCR amplification was first introduced by Saiki *et al.* (1985), and has been widely used for bioanalysis, because it can amplify trace amounts of nucleic acids to a detectable level, although concerns for the fidelity of the representative products are described in an accompanying chapter (Kowalchuk *et al.*, 2007). A brief introduction to the PCR amplification process is as follows. Template DNA molecules are denatured to form two complementary single strands at an elevated temperature (about 95°C). Temperature is then lowered for the annealing step: primers specifically bind to the complementary sequences of the DNA templates (usually 50–65°C). Finally, the temperature is raised to allow polymerase catalyzed DNA extension: the template is typically replicated by a thermostable DNA polymerase at about 72°C. Theoretically, the number of molecules generated by a PCR amplification starting with a single molecule after n cycles is $(1+p)^n$, where p is the probability for a molecule to duplicate, and is usually close to 1 for a good reaction system. A schematic description of PCR amplification is shown in Figure 19.

Microchip-based PCR amplification has many advantages over its conventional counterpart. For example, the costs mostly due to the price of the enzyme will be significantly reduced as the volume scales are down. Additionally, miniaturized systems present less inertial mass to temperature changes, and thus rates for heating or cooling are drastically increased, giving rise to more rapid template amplification. Nowadays, there are two main types of PCR amplification on microchip format: well-based amplification and continuous flow-through-based amplification. Microwell-based PCR amplification was first introduced by Northrup *et al.* (1993) and by Wilding *et al.* (1994). The device

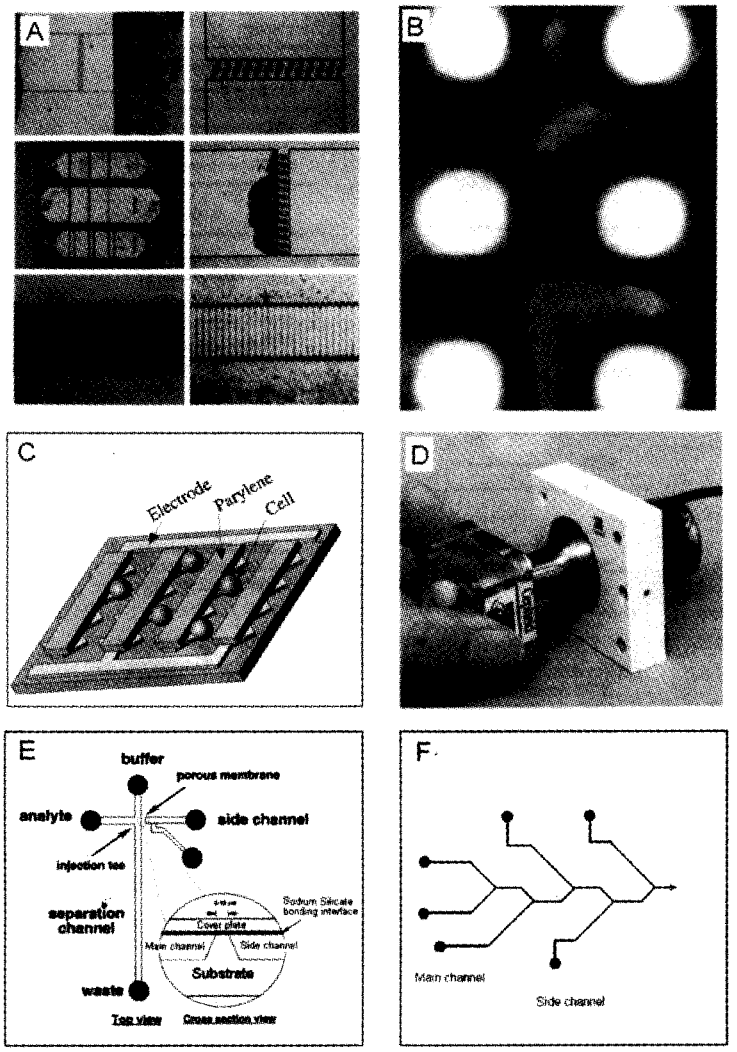


Fig. 18. Various types of preparative microchip created for DNA sample preparation. (A) Microfabricated filters for cell selecting, reprinted from Cheng *et al.* (1998a) with permission. (B) Separation of *Escherichia coli* (white) from human blood cells (red) by DEP on a microfabricated electronic microchip, reprinted from Cheng *et al.* (1998b) with permission. (C) An electronic cell lysis device, reprinted from Lee and Tai (1999) with permission. (D) An ultrasonic cell lysis device, reprinted from Belgrader *et al.* (1999) with permission. (E) Sample concentration using porous membrane structures, reprinted from Khandurina *et al.* (1999) with permission. (F) Microchannels for sample dilution with low error introduction, reprinted from Cheng *et al.* (1998d) with permission.

consisted of a silicon chip with a microwell in which the sample was loaded. The entire chip was heated and cooled to provide the adequate thermo-cycling conditions (Figure 20A). For continuous flow-through-based amplification, the transition times to change temperature depend only on the sample pumping rate

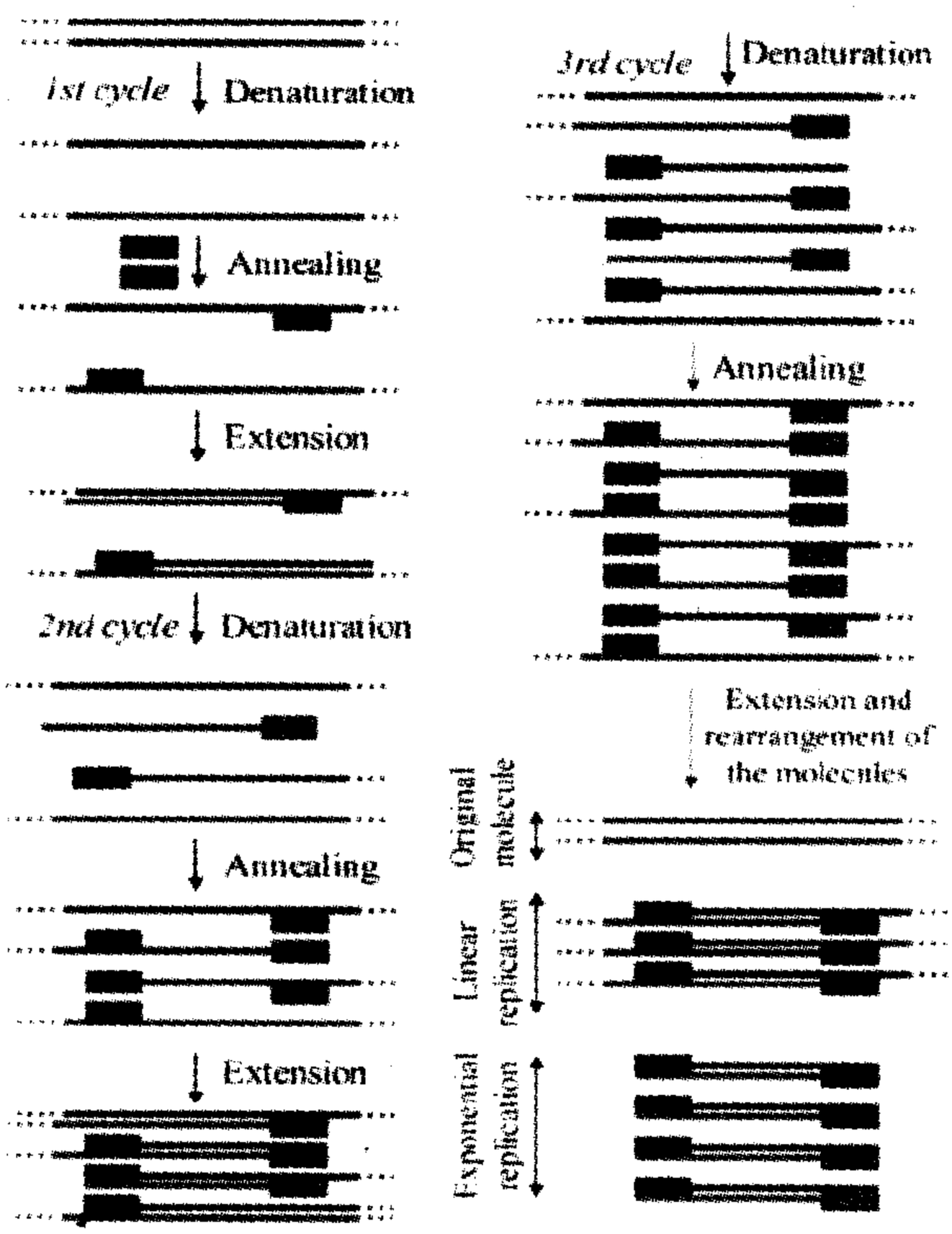


Fig. 19. Schematic illustration of PCR amplification.

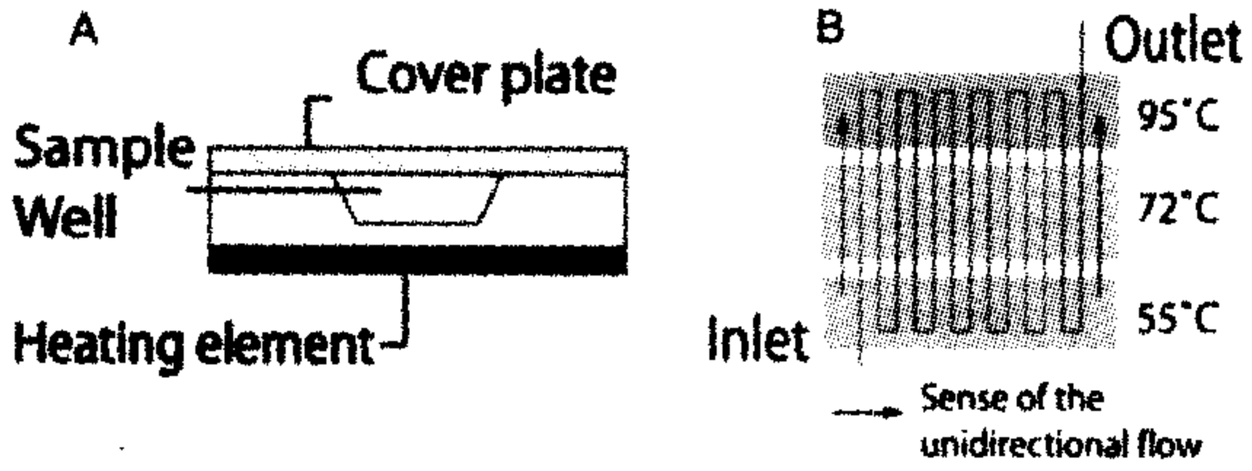


Fig. 20. Schematic view of (A) well-based PCR amplification on a microchip; and (B) continuous flow-through-based PCR amplification on a microchip.

and the time the sample needs to reach temperature equilibrium. It was first introduced by Nakano *et al.* (1994) using capillary-based systems, and the first continuous flow PCR chip was presented by Kopp *et al.* (1998), who fabricated a serpentine channel that passed through three heating zones maintained at constant temperature by copper blocks (Figure 20B).

7.2.3. System integration

To integrate various functional parts to form a miniaturized system, fluid control and manipulation must be realized by using micropumps and microvalves.

To create micropumps on chips, non-mechanical, mechanical–electrical and mechanical forces, such as electro-osmotic pumps (Takamura *et al.*, 2003) and bubble-driven pumps (Tsai and Lin, 2002) have been used. Various types of microvalves have also been microfabricated. For example, Harmon *et al.* (2003) introduced an actuator, which was based on a thermo-responsive hydrogel that shrank or swelled with fluid from a separate reservoir and thereby displaced a PDMS membrane to actuate fluid in the microchannel underneath. More types of microvalves such as check valves, diverter valves and micropipettes were provided by Hasselbrink *et al.* (2002), who employed moving micropistons formed *in situ* by laser polymerization. Some successful demonstrations of total genetic analysis systems are detailed below.

Cheng *et al.* (1998a, 1998c) introduced the first total genetic analysis system that incorporated sample preparation, bioreactions and detection. First, *Echerichia coli* were separated from blood cells using DEP on a silicon chip. An array of 25 individually addressable microelectrodes were microfabricated in two patterns (square-wall and checkerboard); both patterns functioned well (Figure 21A and B). The separated bacteria were retained above the electrodes after the blood cells had been washed off. Then, the isolated prokaryotic cells were lysed electronically by applying a series of high-voltage pulse to release a full size spectrum of nucleic acids. Next, protein was digested by introducing proteinase K. Finally, the released RNA and DNA were transferred onto another electronic microchip, where electronically enhanced hybridization of DNA or RNA was performed for detection of the initial bacterial cells (Figure 21C).

Another development of an integrated miniaturized system for DNA analysis was also introduced Burns *et al.* (1998), in which almost all functional parts were integrated onto one single microchip, this included a nanoliter liquid injector, a sample mixing and positioning system, a temperature-controlled

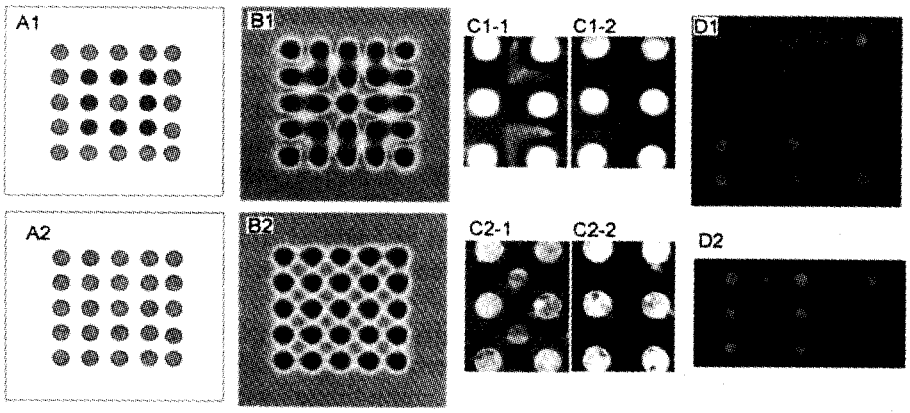


Fig. 21. The first complete total genetic analysis system. A1, A2: square-wall and checkerboard design of electrode array. B1, B2: simulated electronic field generated from corresponding types of electrodes. C1, C2: cells separation by means of DEP using the two electrodes designs. D1: hybridization results of DNA generated by electronic cell lysis. D2: hybridization results of RNA generated by electronic cell lysis. Reprinted from Cheng *et al.* (1998a, 1998c) with permission.

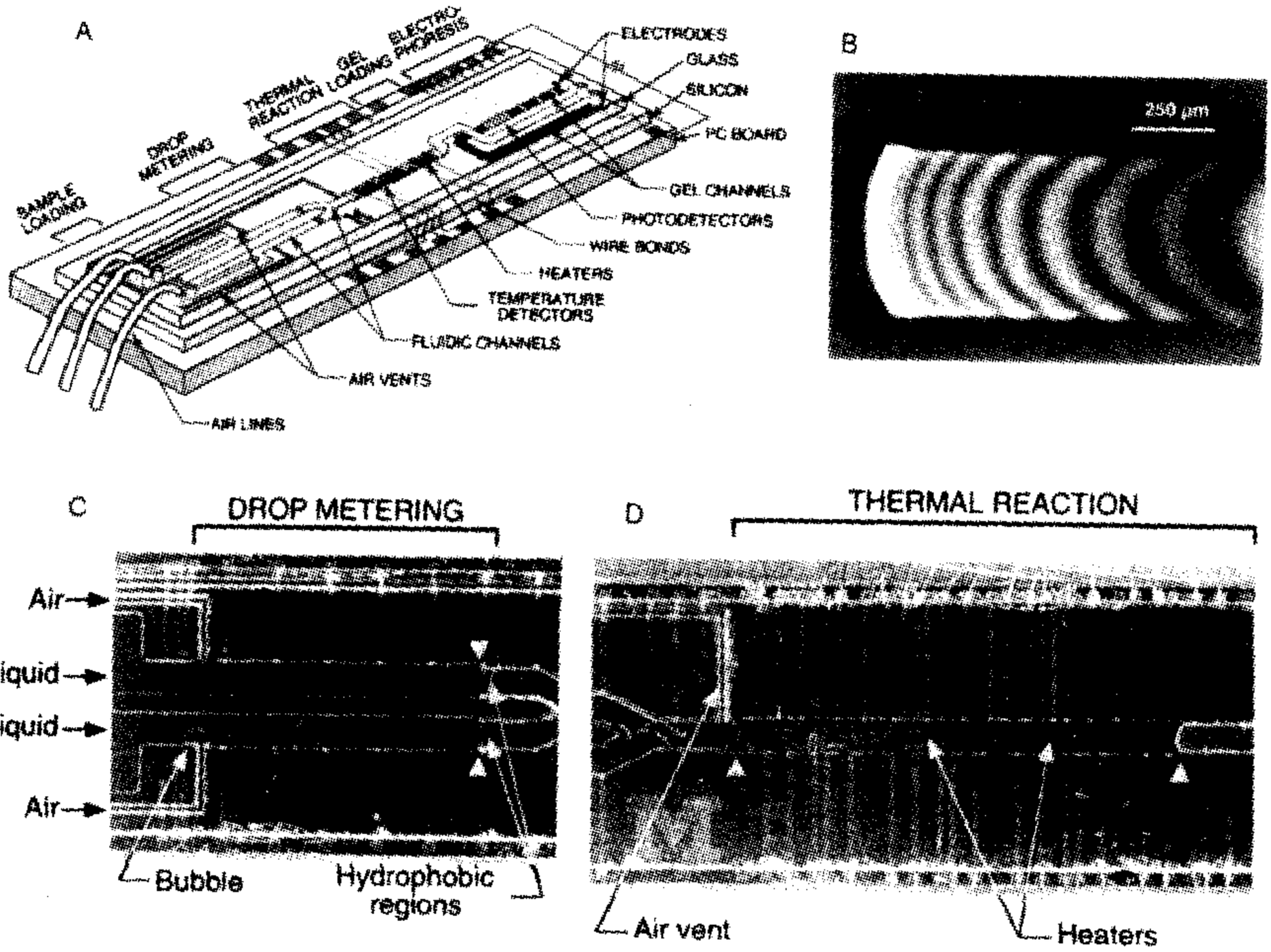


Fig. 22. (A) Overview of Burns's integrated device for DNA analysis. (B) Optical micrograph of a 50 bp DNA ladder separated via on-chip gel electrophoresis, where the separation path length is about 1 mm. (C) Micrograph of the drop-metering region. (D) Micrograph of reaction region. Reprinted from Burns *et al.* (1998) with permission.

reaction chamber, an electrophoretic separation system and a fluorescence detector (Figure 22). Liquid samples were injected via capillary action and were metered by means of an air vent and a hydrophobic patch positioned sequentially along the microchannels. Liquid aliquots with a volume of 120 nL could be defined precisely. The aliquots in different microchannels were then mixed together and moved forward the reaction chamber, where integrated metal heaters and sensors were positioned to facilitate PCR amplification, or enzymatic digestion of the sample. After processing in the bioreactor, the products were subjected to an on-chip gel electrophoresis. *In situ* polymerized polyacrylamide was used as sieving matrix, with an efficient effective separation path length of several millimeters. Diode photodetectors, as well as a quarter-wavelength interference filter, were microfabricated directly beneath the separation channel to facilitate fluorescence detection.

More recently, a fully integrated miniaturized system created by Liu *et al.* (2004) from the Motorola Corporation provided a *tour de force* demonstration of a self-contained DNA analysis. This miniaturized system consisted of micofluidic mixers, valves, pumps, channels chambers heaters and a microarray of DNA sensors. Sample preparation (including magetic bead-based cell capture, cell preconcentration, cell purification and cell lysis), PCR amplification, DNA hybridization and electrochemical detection were each performed on chip with no external pressure source, fluid storage, mechanical pumps or valves required for fluid manipulation (Figure 23).

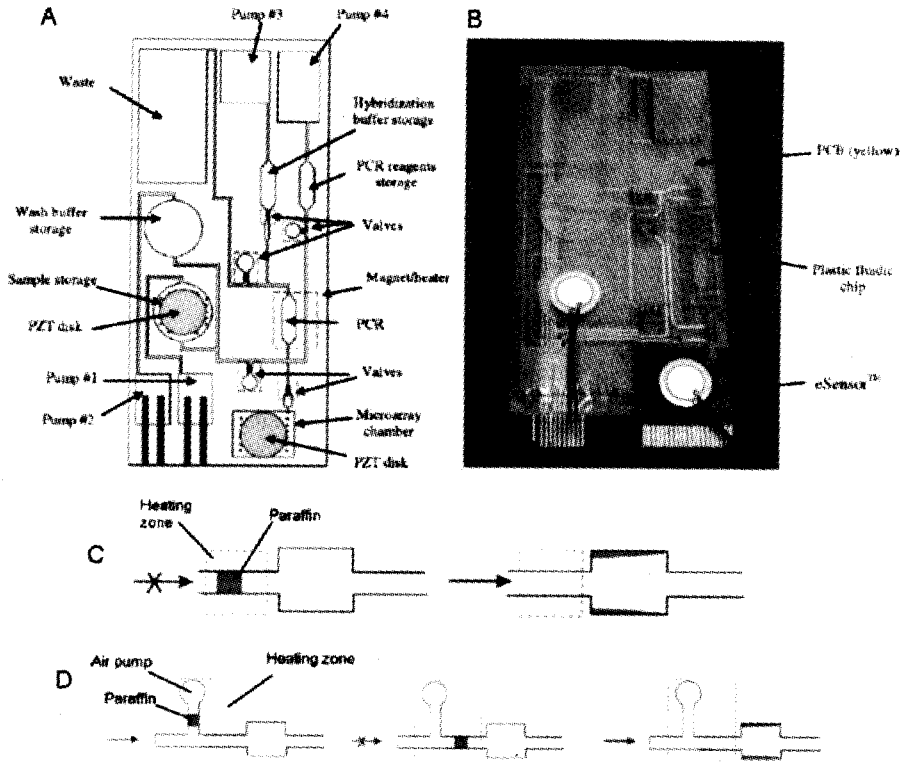


Fig. 23. (A) Design layout and (B) photograph of the fully integrated microsystem for DNA analysis. (C) Design of the close–open and (D) The open–close–open valves employed for fluid manipulation. Reprinted from Liu *et al.* (2004) with permission.

A biological sample requiring analysis (such as blood solution) of a solution containing immunomagnetic capture beads was first loaded into the sample storage chamber, and other solutions including washing buffer, PCR reagents and hybridization buffer were separately loaded into other storage chambers. Cavitation microstreaming generated by vibrating bubbles was used to mix the sample and other reagents in various chambers. Two types of micropumps, electrochemical pumps (driven by the generation of gas by electrolysis of water to move a piston liquid) and thermo-pneumatic air pumps (driven by the expansion of gas via heating to move a piston liquid) as well as paraffin microvalves were employed for fluid manipulation. The detection of pathogenic bacteria from approximately one milliliter of whole blood sample by employing SNP analysis (directly on the diluted blood sample) was successfully demonstrated with this fully integrated microsystem.

7.3. DNA sequencing lab-on-a-chip

As the above section demonstrates, the progressive development of microfabrication techniques and determination of suitable compatible materials is

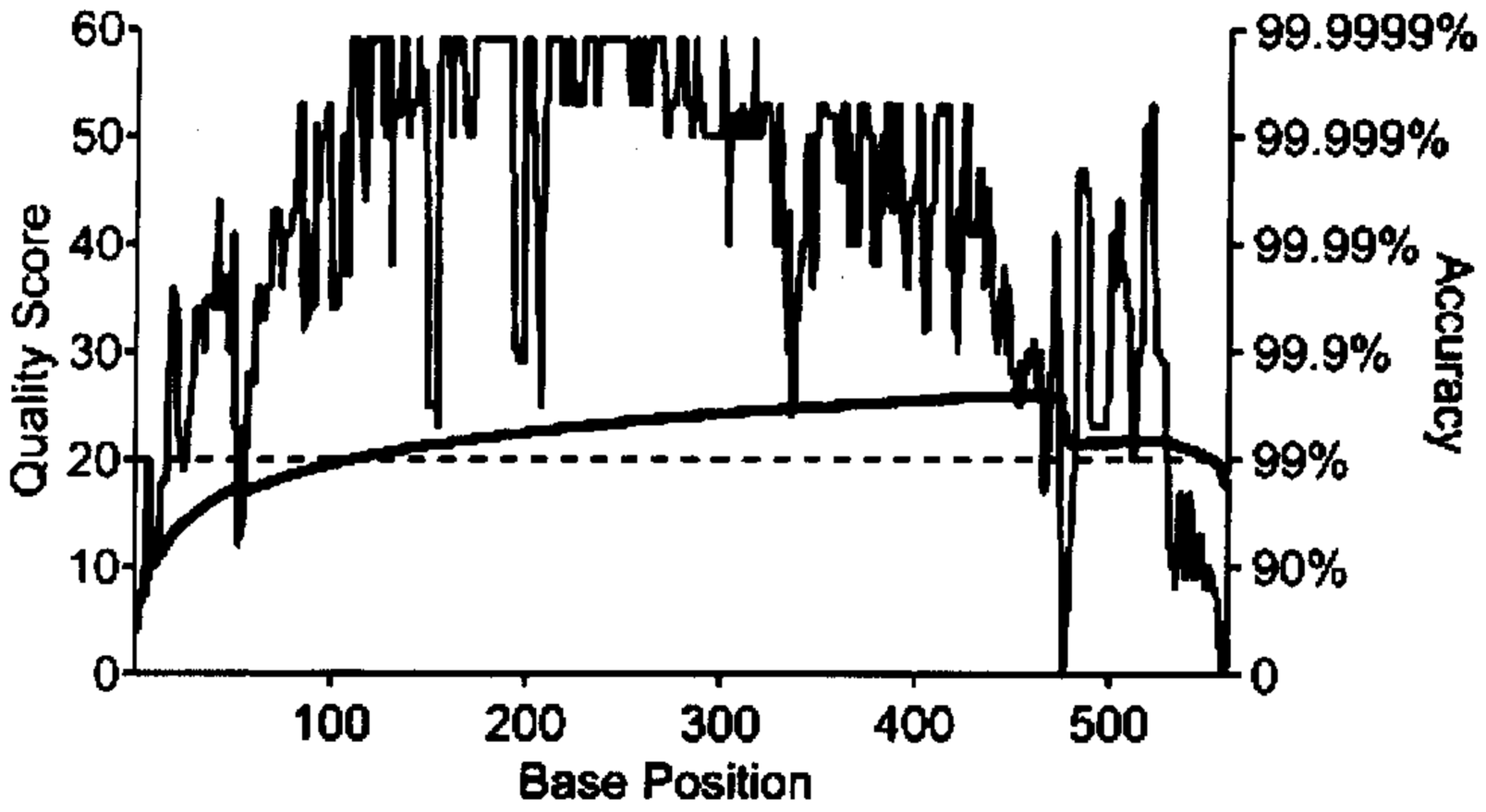


Fig. 24. The base-call accuracies and sequence read length predicted by PHRED. The percent accuracy is related to the PHRED quality score by the relationship $100 \times (1 - P_e)$, where P_e, the probability that the base call is incorrect, is equal to $1/10^{Q/10}$. A one-in-a-hundred error rate is indicated by the dashed line. The gray line plots the PHRED quality scores at each base position, and the black line charts predicted the read accuracy at each base position, $100 \times (\text{base}_i - \Sigma P_{ei})/\text{base}_i$. Reprinted from Blazej *et al.* (2006). Copyright (2006), reprinted with permission from National Academy of Sciences, USA.

allowing the realization of fully integrated microsystems, which are capable of integrated sample manipulation, separation and analysis. The Mathies' group have now recently reported an integrated "lab-on-a-chip" DNA sequencing system (Blazej *et al.*, 2006). It involved the construction and application of an efficient, nanoliter-scale microfabricated bioprocessor that combined all three Sanger sequencing processes of thermal cycling, sample purification and CE into a single analytical device. The design had several advanced and unique features for miniaturization and integration. It used both electrophoretic and pneumatic forces for sample movement and for improved sample transfer through holes into channels. It also used a hybrid glass–PDMS assembly that was necessary for parallel processing, and the multilayer construction also enabled a much greater design complexity and permitted the exchange of materials across fluidic and pneumatic lines. The wafer-scale device was constructed to form a single microfabricated instrument with pneumatic valves and pumps, different 250-nL reactor chambers, affinity-capture purification chambers, and high-performance CE channels. This device was shown to be capable of undertaking complete Sanger sequencing from only 1 fmol of DNA template with reads of > 500 bp. The base-call accuracies and sequence read length predicted by PHRED of a typical sequence are shown in Figure 24. Further advances in lab-on-a-chip electrophoretic sequencing technologies are soon to be expected.

7.3.1. Alternative DNA sequencing technologies

A number of non-Sanger DNA sequencing technologies have recently begun to emerge as alternative contenders for the $1000 per genome prize. None of these technologies use electrophoresis or fragment sizing. Several of these

technologies involve detection of short DNA extension process events (typically a single nucleotide addition at each cycle) catalyzed simultaneously on different DNA templates bound at numerous fixed locations (100,000 to 1,000,000 features) arrayed on a planar "chip" surface. Each extension reaction at each feature is detected by imaging fluorescent dye nucleotides incorporated by each unique template (Edwards *et al.*, 2007; Kumar and Fuller, 2007; Hebert and Braslavsky, 2007) or by CL detection of a linked enzymatic process (pyrosequencing) (Margulies *et al.*, 2007). These technologies are characterized by their reliance on precision microengineering, microfluidics and ultra-precision optics and ultra-sensitive imaging devices. Although rapid progress is being made different aspects of this approach, in the length of sequence read, in the number of arrayed templates, and in efficient data generation with limited amounts of sequencing chemicals, these sequencing technologies are characterized by short reads (30–100 + bp), which at present cannot compete for data utility with the high quality of Sanger sequence reads obtained even on high-throughput microfabricated CE devices. Readers are directed to chapters by Margulies *et al.* (2007) on pyrosequencing, Edwards *et al.* (2007) and Kumar and Fuller (2007) on dye-nucleotide extension cyclic sequencing and to Hebert and Braslavsky (2007) on single molecule cyclic sequencing for fuller accounts of the development of these exciting new sequencing technologies.

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