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Review

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DNA sequencing and genotyping in miniaturized electrophoresis systems

Advances in microchannel electrophoretic separation systems for DNA analyses have had important impacts on biological and biomedical sciences, as exemplified by the successes of the Human Genome Project (HGP). As we enter a new era in genomic science, further technological innovations promise to provide other far-reaching benefits, many of which will require continual increases in sequencing and genotyping efficiency and throughput, as well as major decreases in the cost per analysis. Since the high-resolution size- and/or conformation-based electrophoretic separation of DNA is the most critical step in many genetic analyses, continual advances in the development of materials and methods for microchannel electrophoretic separations will be needed to meet the massive demand for high-quality, low-cost genomic data. In particular, the development (and commercialization) of miniaturized genotyping platforms is needed to support and enable the future breakthroughs of biomedical science. In this review, we briefly discuss the major sequencing and genotyping techniques in which high-throughput and high-resolution electrophoretic separations of DNA play a significant role. We review recent advances in the development of technology for capillary electrophoresis (CE), including capillary array electrophoresis (CAE) systems. Most of these CE/CAE innovations are equally applicable to implementation on microfabricated electrophoresis chips. Major effort is devoted to discussing various key elements needed for the development of integrated and practical microfluidic sequencing and genotyping platforms, including chip substrate selection, microchannel design and fabrication, microchannel surface modification, sample preparation, analyte detection, DNA sieving matrices, and device integration. Finally, we identify some of the remaining challenges, and some of the possible routes to further advances in high-throughput DNA sequencing and genotyping technologies.

Keywords: DNA sequencing and genotyping / Microchannel electrophoresis / Miniaturization / Review
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Abbreviations: CAE, capillary array electrophoresis; **DEA**, *N,N*-diethylacrylamide; **DMA**, *N,N*-dimethylacrylamide; **HA**, heteroduplex analysis; **HEC**, hydroxyethylcellulose; **HGP**, Human Genome Project; **HPMC**, hydroxypropylmethylcellulose; **LPA**, linear polyacrylamide; **μTAS**, micrototal analytical systems; **NEEA**, *N*-ethoxyethylacrylamide; **NHGRI**, National Human Genome Research Institute; **NIR**, near-infrared; **NMEA**, *N*-methoxyethylacrylamide; **PC**, poly(carbonate); **PEO**, poly(ethylene oxide); **PMMA**, poly(methylmethacrylate); **PVA**, poly(vinyl alcohol); **SNP**, single nucleotide polymorphism; **TEG**, Tris-EDTA-glycerol; **TTE**, Tris-TAPS-EDTA

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1 DNA sequencing and genotyping technology in the "post-genome" era

The announcement of the completed human genome sequence in 2003 (on the 50th anniversary of the discovery of the DNA double-helix structure) [1, 2] marked a significant milestone of the Human Genome Project (HGP), and is one of the most important scientific accomplishments in human history [3]. This achievement opens the door to a variety of genomic and proteomic research projects, and the resulting technological revolution will have unprecedented impacts an medicine [4], forensics [5], molecular biology and biotechnology [6], and many other related and even unrelated disciplines. In addition to revolutionizing biomedical research, investments made by the HGP also catalyzed numerous, significant technological innovations in the analytical sciences. The past 10–15 years have witnessed the invention and development of automated, high-throughput DNA sequencing and other genotyping technologies based on microchannel electrophoretic separations, involving the complete transformation of the system platform from thick to thin slab-gels and then to capillaries, and presently to a microfabricated chip format.

Various ambitious goals for the future of genomic research have been put forward that will continue to demand the rapid evolution of high-throughput genotyping technologies toward increased speed and reduced cost [7]. Current technology, based on the fused-silica capillary arrays used in capillary array electrophoresis (CAE), is still costly and relatively slow, and may soon become a significant obstacle to future progress despite its important contributions to the HGP. A next-generation technology will be needed to meet society's ambitious goals, such as the goal set by the National Human Genome Research Institute (NHGRI) to sequence a human individual's genome for under $ 1000 [1]; the burgeoning need for high-throughput and low-cost forensic profiling [8–10]; the growing interest in genetic medicine ("pharmacogenomics") [11], and many others.

Performing sequencing and genotyping on microfluidic platforms can lead to a significant increase in throughput. For example, a DNA sequencing read length of 600–800 bases could be achieved in ~ 25 min using microchip electrophoresis with a separation channel of 20 cm, whereas the same separation requires 1–2 h in a CAE system [12, 13]: hence, the chip provides a fourfold increase in throughput. What is even more attractive, is the substantial cost reduction potentially obtainable with microchip electrophoresis systems. On top of the reduction of overhead cost due to the reduced sample requirements of an ideally miniaturized, fully integrated system, the raw cost per microchannel itself is a major cost saver. In particular, a typical, coated 16-capillary array suitable for DNA sequencing costs about $ 600–800 at present, so a set of six 16-capillary arrays necessary for a 96-capillary instrument costs at least $ 3600. As we know that the chemistry of the typical glass substrate for chips is essentially the same as a fused-silica capillary, we could safely assume that a glass chip could be at least as effective and durable as a fused-silica capillary array. High-quality glass wafers for the devices, made from borofloat glass, can be purchased at relatively low price (~ $ 50–100 for a 6-inch wafer); adding on the labor and materials costs for fabrication and surface modification, a typical microfabricated CAE device should cost at most $ 400, representing only ~ 10% of the cost of the capillary arrays used in CAE. In addition, the procedure and the materials needed for fabricating a 96-channel chip and a 384-channel chip are the same; hence, no significant increase in the materials and fabrication cost should be anticipated when developing even higher throughput devices. Plastic chips (made from, *e.g.*, poly(methylmethacrylate) (PMMA)) promise to be one or two orders of magnitude cheaper than this. This makes the technology very affordable, and widespread applications such as clinical genotyping more feasible. Hence, the

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advantages of developing and using microfabricated DNA sequencing and genotyping devices are obvious and desirable. Over the past few years, significant efforts have been put into pushing this microfluidic technology forward, to realize the advantages of microchips over the present CAE systems for genetic analyses. A few prototypes have been demonstrated in academic and industrial laboratories, which illustrate that there are no prohibitive technological limitations in using this technology to directly replace CAE systems [14, 15]. However, we still have not seen major commercialized microfabricated DNA sequencing and genotyping platforms, except for a few devices marketed by Agilent Technologies (Palo Alto, CA, USA) and Caliper Life Sciences (Mountain View, CA, USA) for less exacting tasks such as double-stranded (ds) DNA and RNA fragment sizing. While we agree that there are still several hurdles to be jumped, it is unclear as to why the commercialization of microfabricated DNA sequencing and genotyping platforms has been taking so long, as opposed to the case of CAE. Our opinion is that the commercial investment in developing sequencing chips has been sporadic, advancing in fits and starts as companies working in this area were acquired and re-organized, or folded in bad economic times. However, in the process of reviewing the developments in this area, we would also like to seek possible answers as to what technological hurdles have so far prevented the routine and widespread application of this promising technology.

We focus this review on advances in miniaturized electrophoretic separation systems for genetic analyses, and the implications of these more efficient devices for the immediate future of the life sciences and medicine. Several important genotyping and genetic variation detection techniques are discussed to emphasize the continuing, central importance of electrophoretic DNA separations. The state of current DNA separation technology, *i.e.* CAE, for high-throughput DNA sequencing and genotyping research is briefly reviewed. Progress in the development of miniaturized/microfabricated genotyping devices is discussed thoroughly. We focus this review on microfabricated devices utilizing electrokinetic phenomena (electroosmosis and electrophoresis) as the main transport mechanism; microarray chips using DNA hybridization as the probing method are not discussed. While an interdisciplinary approach will eventually be required to meet the technical challenges inherent to the development of integrated microfluidic devices, we critically evaluate the achievements made by researchers from different areas that have led to improvements in chip design and fabrication, sample preparation, analyte detection, DNA separation matrices, and device integration, *etc*. We conclude the review by identifying some remaining challenges and discussing the outlook for this technology to fulfill its promise, which has been hailed now for more than a decade.

2 Genetic analyses that rely on electrophoretic DNA separations

Determining the sequence of the human genome is only the first step toward understanding genetic inheritance and the mechanisms of essential life processes, however, large-scale DNA sequencing necessarily has been the focus during the first decade of the HGP and in other large genomic research projects. While DNA sequencing is the most important type of genetic analysis, it is well recognized that other genotyping and mapping techniques are necessary for the efficient extraction of useful data from the human genome. The most common genotyping techniques presently under research and development are single-nucleotide polymorphism (SNP) detection, single-stranded conformation polymorphism (SSCP), heteroduplex analysis (HA), and short tandem repeat (STR) analysis; in these methods, resolving DNA fragments according to differences in size and/or conformation is the most critical step. A dramatic increase in the efficiency and sensitivity of various analytical chemistry tools has led to prototypes of the first potentially high-throughput and low-cost genotyping devices, which will in principle allow the general public to benefit soon from the fruits of the HGP.

2.1 DNA sequencing

The present state-of-the-art method for DNA sequencing relies upon the Nobel Prize-winning Sanger dideoxy chain termination reaction [16], which is based on the controlled interruption of the enzymatic replication of a ssDNA template by a DNA polymerase. In its modern incarnation, the base sequence of DNA is determined by fragmenting the genome into small pieces, using the Sanger reaction to produce a "ladder" of template-complementary DNA fragments that differ in length by one base and that bear unique fluorescent labels according to their terminal nitrogenous base, electrophoretically separating these fragments on the basis of chain length with single-base resolution, detecting the base-specific labels, and then, computationally reassembling the fragments of sequence into their original order. Electrophoretic DNA separation is virtually always carried out in a polymeric sieving matrix to compensate for DNA's constant charge-to-frictional coefficient ratio that leads to its size-independent electrophoretic mobility in free solution [17]. This sieving matrix can be either a cross-linked gel or an entangled

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polymer solution. The current high-throughput DNA sequencing technology adopted in most commercial instruments relies on electrophoretic DNA separations carried out in arrays of fused-silica capillaries filled with an uncross-linked polymer solution, with automated, *in situ* "finish-line" detection of fluorescently labeled ssDNA fragments. In practice, a single electrophoretic separation in a good matrix yields at most about 800 bases of high-quality, error-free DNA sequence per sample and per microchannel (*i.e.*, a "read length" of 800 bases), with a read length of 540–650 bases being much more typical. A human gene is commonly comprised of 100 kilobases (kb) or more of DNA, therefore, due to technological read-length limitations related to the physics of matrix-based electrophoresis and the properties of the matrix materials [18], the DNA must first be cut into overlapping short segments, approximately 1 kb in length. These small segments can then be sequenced directly, and the pieces assembled by computer into the final sequence. Achieving high-resolution DNA separations with extended read length is one of the primary goals in the development of robust, low-cost DNA sequencing technologies. By extending the read length of each single electrophoretic separation, the cost for *de novo* DNA sequencing can be reduced substantially and the number of templates needed to sequence DNA contigs at a given redundancy will be lowered, and finally sequence assembly will be faster, cheaper, and easier [19].

2.2 SNP, SSCP, and single-base extension (SBE) analyses

With the availability of the human genome sequence, the next major endeavor of the NHGRI is to construct a haplotype map to identify and understand natural genetic variation. The establishment of the International HapMap Project fosters the discovery of genetic factors that contribute to common ailments such as diabetes and cardiovascular disease [1, 20]. Genetic or DNA sequence variation is the fundamental raw material for evolution. It is also the basis for variations in risk among individuals for numerous medically important, genetically complex human diseases. Among the many existing techniques, SNP mapping is arguably the most important means of bringing the benefits of the HGP to the general public regarding disease-related mutations or sequence variation detection. SNPs are single-base pair changes or deletions in a natural DNA sequence. There is an estimated number of 2–3 million SNPs that differ between any two individuals' genomes ($\sim$ 1 every 1000 bp) [21]. While it is still far, far too costly to determine an individual's entire genome sequence for medical purposes, scanning the genome haplotype by haplotype would be only 5% of the cost of sequencing the whole genome [20], and a preliminary set of useful genomic markers has been identified that can be related to an individual's disease susceptibility. Most SNP scanning technologies utilize oligonucleotide hybridization [22–29]; however, the use of DNA hybridization chips requires some prior knowledge of the sequence of interest, typically uses large amounts of synthetic DNA probes for screening, and is done using relatively expensive array-based technology.

Alternative mutation detection techniques have been developed based on electrophoretic size-and/or conformation-based DNA separations, such as SSCP and HA [30–32], or tandem SSCP-HA [33, 34], SBE [35, 36], and polymorphism ratio sequencing (PRS) [37]. Among these techniques, SSCP (which involves a mobility shift assay combining mutated DNA with known wild-type DNA, and creating single-stranded conformers by denaturing and snap-cooling) alone or in conjunction with HA (which involves analyzing the combined hybrid duplexes of both mutant and wild-type DNA), are two very promising approaches to creating a simple, robust, low-cost, and high-speed electrophoretic method to screen for genetic alterations related to disease [38]. These and other mobility shift-based techniques are being implemented on prototype "lab-on-a-chip" diagnostic platforms aimed at eventual clinical applications [27, 39].

2.3 STR analysis

STR analysis is aimed at characterizing another common genetic variation, which is highly polymorphic in nature among individuals. STRs are short stretches of repetitive DNA sequence that are distributed throughout the genome. Each genetic locus consists of 7–20 repeats of a specific 2- to 7-base sequence. Alleles at a locus are identified by electrophoresis of the PCR-amplified product. Ideally, single-basis resolution of 100–500-bp fragments will best be able to precisely and unambiguously resolve the allele lengths of STRs, although in some cases 2 to 3 bp resolution is sufficient. Among various advances in STR typing technologies, the development of a PCR-based method, with the products analyzed by CE, has gained the most popularity [40]. STR analysis has developed into an important tool in forensic analysis [41]. It also finds important applications in linkage analysis, gene mapping and discovery, paternity testing, evolutionary studies, and clinical diagnostics [42, 43]. CE and CAE have been the technology of choice for high-throughput STR analysis; the ABI Prism 310 and 3100 Genetic Analyzers from Applied Biosystems (Foster City, CA, USA) along with associated kits have become the commercial standard. Multicolor fluorescence detection systems have been developed for accurate, multiplexed STR

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genotyping in which single-base resolution of ssDNA up to about 400–500 bases using entangled polymer solutions allows reliable microvariant allele detection. Improvements leading to higher throughput, better sensitivity, and automation have been the recent focus of the forensic technology research community. Microfabricated genetic analysis systems are likely to be the major platform utilized for the next generation of high-throughput, portable devices for reliable forensic investigations, and as such are the subject of intense research [14, 44–46].

3 Miniaturized systems for genetic analyses

3.1 CAE: from large-scale instrumentation to microfabricated devices

Technologies to enable miniaturized DNA electrophoresis within fused-silica capillaries (50–75 μm ID) have been under development since the late 1980s [47], and were vigorously pursued by a number of research groups and industrial scientists throughout the 1990s [48, 49]. The large surface area-to-volume ratio of a capillary tube allows effective dissipation of Joule heat, allowing the voltage limitations that are imposed on slab-gel electrophoresis to be surpassed greatly. With the use of a higher electric field (within the limits of avoiding the onset of biased reptation [50]), higher DNA separation speed is attained in microchannel systems. The reduced reagent and sample consumption allowable in CE (nL as opposed to μL) could potentially drive down the overhead cost in genomic research centers, although technologies to take advantage of this are not yet widely implemented. After the commercialization of multiplexed CAE systems, this technology became the leading, standardized method, and has successfully and effectively replaced slab-gel electrophoresis over the past 5 years. The development of CAE systems, consisting of 8, 16, 96 or even 384 capillaries, now allow high-throughput, parallel DNA analyses in arrays of fused-silica capillaries. Pioneered in NIH-NHGRI-supported academic laboratories including those of Mathies *et al.* [51, 52], Yeung *et al.* [53, 54], Dovichi *et al.* [55], and in Japan by Kambara *et al.* [56, 57], technology for CAE has been successfully developed and commercialized by Beckman Coulter (Fullerton, CA, USA), Amersham Biosciences – GE Health Care (Sunnyvale, CA, USA), Applied Biosystems (Foster City, CA, USA), SpectruMedix (State College, PA, USA), and others. These high-throughput, expensive (\$ 80 000–\$ 320 000) sequencing and genotyping systems are the major tools used in genome centers and in the genomic research communities. Numerous reviews have been published to recognize the outstanding contribution of CAE to genomic analyses, which discuss the technological aspects of CAE in detail [58–66]. To date, the best published DNA sequencing performance achieved in a capillary-based instrument involved the use of ultrahigh-molar-mass linear polyacrylamide (LPA; > 10 M g/mol), prepared by inverse emulsion polymerization, to deliver up to 1000 bases of high-quality sequence in about 1 h [67, 68] or 1300 bases in 2 h [69] under highly optimized conditions (including optimized polymer molar mass distribution, matrix formulation, electrophoresis temperature, electric field, meticulous sample preparation and purification, and a powerful base-calling algorithm). A read length of 500–800 bases in 2 h is presently more typical for the CAE analysis of "real" sequencing samples on a day-to-day basis. Although this represents a major improvement in throughput compared to the slab-gel electrophoresis systems, which were the only available sequencing technology for decades and widely used until 1999, higher throughput and capacity are desirable to meet the goals of various sequencing and genotyping endeavors. To this end, microchip electrophoresis technology is being developed as the next standard or "workhorse" technology.

Further miniaturization of DNA separation technology from CAE systems to microfluidic devices is the present focus of cutting-edge physical and analytical chemical engineering research. Point-of-care diagnostic devices will no doubt also be developed with miniaturized technologies. Across several disciplines, tremendous efforts have been invested in developing microfluidic "lab-on-a-chip" devices, which have the ultimate goal of integrating the entire sample preparation and analysis process on a microfabricated substrate, or micrototal analytical system (μ-TAS) [70–75]. The advent of microfabricated bioanalytical technologies will revolutionize genotyping and clinical diagnosis in the same way that silicon microfabrication technology revolutionized the computer and electronic industries a quarter-century ago. While bioanalytical microchips can be classified into two categories: microarray (hybridization) chips and microprocessing (electrokinetic) chips, we focus exclusively on discussing the latter, which utilize electrokinetic transport (a combination of electrophoresis and/or electroosmosis) of charged biomolecules for high-resolution analysis. There are obvious advantages in terms of analysis speed and cost, as discussed above [12, 13, 15]. In addition, the shift from a one-dimensional (cylindrical capillary) to a two-dimensional (planar chip) separation space also allows for the design of complex microchannel layouts and staged functions. More complex layouts have two major advantages over a one-dimensional format. First, the use of cross-channels for sample injection allows for the injec-

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tion of narrow, well-defined sample zones, significantly reducing the separation distance required for adequate resolution of DNA sample peaks (by a factor of ~ 5) [12]. Second, the nearly limitless chip formatting options available allow for the design of integrated systems that may include multiple functions, potentially leading to a practical “lab-on-a-chip”. For example, a prototype chip, as shown in Fig. 1a, was developed by Burns *et al.* [76] that integrated dsDNA digestion, PCR amplification, dsDNA separation, and detection, with precise heat and mass transfer control in a device of portable size. A few integrated chip devices have been demonstrated by the Quake group, designed for cell sorting and analysis (Fig. 1b) [77], integrated PCR and dsDNA analysis [78, 79], as well as a microfluidic platform for large-scale integration [80]: all of these aspects of integration are only practically feasible in microfabricated devices. In the following sections, we will discuss extensively the development of various technological elements taking us toward the creation of a practical, fully integrated microfabricated sequencing and genotyping device, and identify some of the remaining challenges to be overcome in the course of achieving widespread, commercial application of this technology.

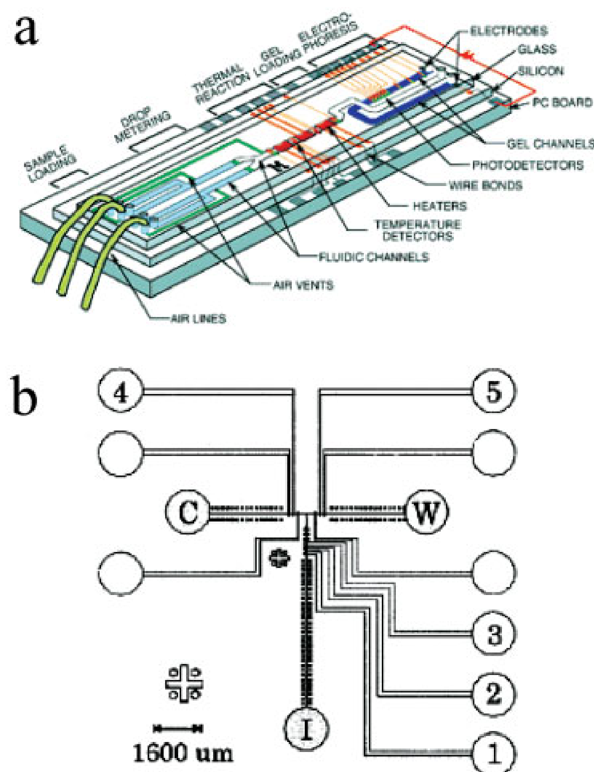

Figure 1. Prototypes of integrated microfabricated platforms for (a) PCR and DNA sizing with temperature and flow controls. Reprinted from [76], with permission; and (b) cell sorting and analyses. Reprinted from [77], with permission.

3.2 Microchannel design for electrophoresis chips

When working in the diffusion-limited regime, DNA separation resolution scales with the square root of the channel length in both capillary and chip electrophoresis [81]. While DNA sequencing is routinely carried out in capillaries with an effective separation distance of 40 cm or more, it is difficult and does not make sense to use such a long channel length in microchip electrophoresis. However, it has been shown that a relatively long separation channel is required for long-read DNA sequencing by microchip electrophoresis: while a read length of 200 bases can be achieved in a 3 cm long channel [12], the read length is significantly increased (to ~ 500 bases) in a 7 cm long channel [82]. Further investigations on extending DNA sequencing read length with longer separation channels have been carried out, culminating in the achievement of a 580 base read in an 11.5 cm long channel [83] and a read length of 800 bases in a less practically significant 40 cm long channel [84] fabricated on glass substrates. It is not trivial to take advantage of the high-speed analysis provided by microchip electrophoresis and the high-resolution DNA separation obtained in a long separation channel simultaneously. One obvious way to achieve the necessary separation distance and high-speed analysis without sacrificing throughput is to fabricate a dense array of long separation channels on a single device. Electrophoretic devices comprising of 16, 96, and 384 channels have been fabricated on 6 inch glass wafers [85–89]. In these devices, turns in the channels are necessary to provide the necessary separation length and accommodate all of the channels on a single planar substrate. Turn geometry has been studied both theoretically [90] and experimentally [91] to reduce turn dispersion and minimize the turn-induced resolution loss. A tapered turn has been found to substantially reduce turn-induced band-broadening. Several parameters including the radius of curvature of the turn, the tapering length, and the degree of tapering were optimized [91]. To summarize the results of these studies, high-resolution DNA separation can be achieved in channels with a small radius of curvature (~ 250 μm), a short tapering length (~ 55 μm), and a large tapering ratio (4:1 separation channel width to turn channel width) [91]. The experimental findings agree well with theory, with respect to the dependency of resolution an different turn geometrical parameters [90].

A microfabricated DNA sequencing device consisting of 96 channels fabricated on a 6-inch glass wafer with this type of tapered turned is shown in Fig. 2. This innovation, along with other auxiliary tools such as a high-speed, rotary LIF scanner [92, 93] and a specialized, high-pressure sieving matrix loader [94], were the key in enabling high-throughput genetic analyses of ssDNA in a single

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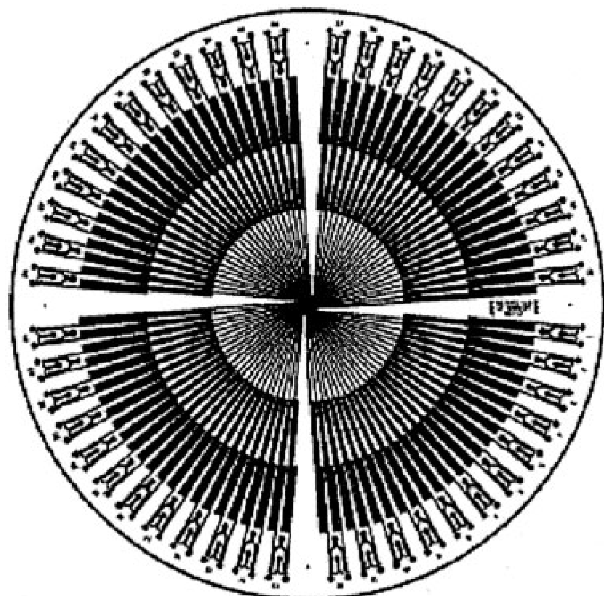

Figure 2. Schematic diagram of microfabricated DNA sequencing device with 96 channels fabricated on a single substrate of 6 inch in diameter. Reprinted from [88], with permission.

electrophoresis device. However, so far only the Mathies group has been routinely applying this technology for multiplexed sequencing and genotyping. In order to accommodate a dense array of separation channels with extremely fine features of the optimized turn geometry, the requirements of the resolution of the fabrication process also become more challenging, and chip assembly tasks become more exacting and difficult, reducing the yield of functional devices when this assembly is done manually. In addition, loading viscous separation matrix into long, periodically constricted channels is challenging. For example, a 384-lane microchip, which consists of separation channels that are 15 μm deep and 60 μm wide with an effective separation length of 5 cm, has only been applied for mutation detection, with the use of a relatively low-viscosity hydroxyethylcellulose (HEC) solution for dsDNA separation [89]. Demonstrations of chip-based DNA sequencing, which requires the use of substantially more viscous solutions of high-molar mass polymers, have so far been restricted to a 96-lane microchip, which has wider channels of 30 μm deep and 200 μm wide for easier pressure-loading of the separation matrix [88].

3.3 Fabrication technologies for glass and plastic devices

Although the first miniaturized analytical devices were developed more than two decades ago [95], they were not widely recognized for their potential use in biomolecule separations until the 1990s [74]. Earlier miniaturized analytical devices were applied to more "traditional" chemical analyses of small molecules, such as gas chromatography [95] and chemical sensing [96]. Silicon or glass (such as borofloat or soda Lime) were the substrates of choice due to the ease of adopting the micromachining methods developed in the silicon microprocessor industry to these materials. When these devices were first applied for biological analyses in the early 1990s, silicon and glass substrates were usually manufactured by standard microfabrication technology. Microchannels and other features are defined on the glass wafers using photolithography and etched by wet chemical techniques [97]. However, microfabrication of microfluidic devices made of glass by these methods for sequencing and genotyping is relatively expensive and time-consuming, and involves multiple steps using harmful chemicals. The need for specialized facilities for fabrication prohibits the widespread use of this technology by researchers. Also, the accessible microchannel geometries are usually limited by the isotropicity of the etching process.

The use of plastic and elastomeric microfluidic devices promises lower manufacturing costs, and could allow the creation of disposable and adaptable genotyping devices. In addition, the use of a polymeric chip substrate allows the incorporation of useful functionalities for microfluidics such as microscale valves and pumps [76, 80, 98–100], which will be key to the successful development of integrated devices. The development of microfabricated plastic bioanalytical devices has been led by the Soper group, Locascio and co-workers, and a few others. However, technical issues such as the high background fluorescence signal generated from the polymeric substrate and difficulties with respect to heat dissipation should be considered when designing plastic microfluidic devices. In addition, quality control over the production of most commercially available polymeric materials, to the level required for bioanalytical devices, is questionable: the chemical and physical properties of the plastics can fluctuate widely within the same batch of materials, as well as batch to batch, making it difficult to standardize the chip manufacturing process. Also, the compatibility of biological samples with plastic substrates (or lack thereof) will be a major determining factor of the ultimate usefulness of the chips [101].

Several different types of polymeric substrates have been used in microfluidic devices for DNA analysis, including PMMA [27, 102], poly(dimethylsiloxane) (PDMS) [103–105], poly(carbonate) (PC) [106], poly(olefin), and a cycloolefin copolymer (COC) [14]. Novel fabrication methods have been developed to enable the processing

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of substrates other than silicon and glass; a thorough review on polymer microfabrication techniques is presented by Becker and Gärtner [107]. The most common manufacturing method used to make polymeric microchips is either injection molding [108] or hot embossing [109]. During an injection molding process, a master mold is created, one surface of which contains a pattern of interconnected ridges. In the second step, called replication, the pattern of ridges is imprinted onto a plastic sheet, forming the pattem of connected channels. The capillary networks are then enclosed with a top layer of a plastic film, using thermal or adhesive bonding. The major drawbacks of injection molding are that high pressure and temperature are required. It is well-documented that the properties of the polymeric materials are extremely sensitive to environment, and especially vulnerable to pressure and temperature fluctuations, hence maintaining precise control over the chip dimensions becomes difficult with this processing method. Also, the microfabricated silicon templates experience large temperature and pressure fluctuations during process cycles, which limit the lifetime of each silicon template to about only ten process cycles [110].

Hot embossing is a more straightforward method that is applicable for lower-cost mass production. It involves the use of a micromachined, three-dimensional inverse of the microchannels master, which is mounted in the embossing system together with the planar polymer substrate. The silicon master and polymer substrate are then heated separately in a vacuum to above the glass transition temperature, T_g, of the polymer material. Once the desired temperature is reached, the silicon master and the polymer substrate are brought together and compressed at 0.5–2 kN/cm^2. The silicon master and the polymer substrate are then pulled apart, and the microchannel and reservoir systems are sealed with lamination [111]. Hot embossing allows the production of microfluidic devices with a smoother internal microchannel surface and a prolonged lifetime of the mold; however, since the processing of polymer materials above T_g is involved, dimensional control over the chip features is needed, due to the relaxation of the polymer upon cooling, to reduce device-to-device variation. A PMMA-based genotyping device fabricated by hot embossing has been demonstrated for dsDNA analysis with fluorescent detection based on infrared (NIR) excitation (discussed below) [112]. This particular example has illustrated that the ability to produce a high-aspect ratio microchannel by hot embossing allows greater sample loading to increase S/N, while at the same time minimizing electrophoretic zone-broadening with the narrow channel. To alleviate the adverse effect of temperature fluctuations, PMMA microfluidic devices have also been fabricated by room-temperature imprinting [110]. In this approach, the channels are imprinted onto the PMMA slides by applying pressures of up to 2700 psi, and then the open channel is sealed with a piece of PDMS sheeting. This technology is applicable for the creation of microchannels up to 100 μm deep. The channel dimensions and morphology, as characterized by interferometry, were found to be uniform and reproducible [110]. Although devices made in this way have not yet been applied for genetic analysis, this technology has the strong potential to be adaptable for low-cost, mass-produced manufacturing of microfluidic chips, as the expensive silicon template was found to be much more durable when used in this way.

In a different approach, laser ablation has been used as a direct method to create microchannels in plastic chips without the need for master fabrication, although the fabrication throughput is limited, as each device is manufactured individually [113, 114]. The method involves the use of an UV excimer laser to "burn" the microchannels onto the polymer substrate, moving in a predefined, computer-controlled pattern. The channel can be then sealed easily with a low-cost film lamination technique [114]. Microfluidic devices have been fabricated on a number of substrates using this method, such as poly(styrene) (PS), PC, and poly(ethylene terephthalate) (PET) [114]. Laser ablation has been found to have negligible effects an the surface properties of the substrates, as studied by X-ray photoelectron spectroscopy for characterization of the surface chemistry, and current-monitoring mobility measurements for EOF characterization [106]. This is another approach that could be used to fabricate high-performance sequencing and genotyping devices at relatively low cost. But so far, this method has only been employed to manufacture devices for chemical analysis [114]. A number of other substrate-specific fabrication techniques have been investigated to facilitate the mass production of low-cost polymer microfluidic devices for genetic analysis. For example, X-ray lithography has been applied to the fabrication of PMMA microchips: this approach is reported to be advantageous because of the simplicity of the fabrication instrumentation, and the flexibility it allows in creating different channel topographies [115].

While PMMA and PC have been widely used, PDMS possesses a number of desirable properties, such as optical transparency, electrical and chemical inertness, and elastomeric characteristics for easy fabrication, making it in some ways attractive as a microfluidics substrate [116], and a number of PDMS devices have been successfully fabricated for different miniaturized biological analyses including protein and dsDNA separation, and

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cell-sorting and manipulation, as recently reviewed by Sia and Whitesides [105]. PDMS microstructures are mainly fabricated by "soft lithography", an approach which has been described in detail elsewhere [103]. Alternative, bottom-up approaches for the fabrication of 3-D micro-channel systems in PDMS have also been demonstrated [117]. Although the 3-D devices fabricated have not yet been tested for electrophoresis applications, this approach holds promise for the low-cost construction of microfluidic systems comprising PDMS, and the inherently adhesive properties of PDMS allow for easy device assembly. Nonetheless, several technical issues need to be resolved for the use of PDMS-based genotyping devices to be practical. The strongly hydrophobic character of PDMS can lead to severe wall-analyte interactions, especially protein-PDMS interaction and ssDNA-PDMS interaction [118, 119]. Also, the elastomeric character of PDMS limits its shelf life, as PDMS may deform easily under ambient conditions, potentially leading to deformation of the microchannels from the desired geometry.

Another interesting idea is to develop hybrid devices that consist of more than one type of material. The use of hybrid devices is a clever approach to allowing integration of different operations, however at the same time, it requires advanced fabrication and packaging techniques for device construction. As pioneered by the Quake group [77], hybrid devices consisting of a layered inorganic substrate (a silicon wafer) and organic elastomer (PDMS) are used to perform multiple functions on an integrated chip. In a recent publication from the Mathies lab [120], an integrated device, which can perform PCR, CE with electric field control and fluorescence detection, and also includes integrated heaters and temperature sensors, as well as PDMS-membrane valving to control analyte transport, has been demonstrated for pathogen/infectious disease detection. In this innovative design, PDMS is fabricated as membrane valves sandwiched between a glass PCR chamber and the glass CE channel. This design allows the precise control of both process unit operation and analyte transport, and hence an integrated, high-performance device is possible. The use of glass microchannels allows high-resolution dsDNA separation and sensitive detection. Chip design and detection instrumentation would need to be optimized to perform high-resolution sequencing separations of ssDNA, which are much tougher to achieve than dsDNA separations.

3.4 Surface modification methods for microchannel inner walls

The resolution of DNA separation is strongly affected by the surface properties of the capillary and the microchip substrates that form the microchannel. Several surface modification strategies, in both capillary and micro-fabricated devices, have been developed to eliminate electroosmotic flow (EOF), and to minimize the DNA-surface interactions that are detrimental to the analysis by causing peak-broadening. These developments have been found to be one of the most critical issues in realizing high-resolution DNA separations in miniaturized systems. Below, we review several common strategies for surface modification in capillary wall and microchip substrates.

3.4.1 Capillary wall coatings

In an uncoated capillary, residual charges an the silica surface induce a bulk flow of solution having a flat velocity profile towards the cathode, slowing down DNA migration, which leads to increased run times and potentially increased molecular diffusion and dispersion. An effective coating strategy producing consistent results is not only important for DNA fragment sizing [121] and long-read DNA sequencing [122], but is also critical for accurate and reliable mutation detection because DNA analytes with ssDNA regions are more hydrophobic in nature and have a higher tendency to "stick" to the wall's surface [33, 123]. In CE, the silanol surface can be modified by covalent coating with small and large molecules chemically bonded to surface silanols or immobilized *via* a silane reaction as films of various thicknesses on the capillary wall. In particular, covalently bound poly(vinyl alcohol) (PVA) [124] or LPA [125] are commonly employed to control the EOF and reduce nonspecific adsorption of the analyte. On the other hand, "dynamic", or physical adsorption of water-soluble, neutral polymers has been pursued as a quicker, cheaper, and less complicated way to modify the capillary surface. Along related lines, so-called "self-coating", adsorptive polymer matrices have been developed that can be used not only for DNA sieving but also as dynamic coatings, such as poly(dimethylacrylamide) (PDMA) [126, 127], poly(ethyl oxide) (PEO) [128], and poly-*N*-hydroxyethylacrylamide [122] (discussed in the later section). A thorough investigation of the critical parameters that influence the effectiveness of a dynamic wall coating for fused-silica capillaries was undertaken by Doherty *et al.* [129]. Polymer hydrophobicity is a key parameter that must be fine-tuned to promote strong polymer adsorption, to produce a coating that has a thickness greater than the Debye-Hückel double layer, and that also minimizes analyte-wall interactions. Specifically, while copolymers of *N,N*-dimethylacrylamide (DMA) and *N,N*-diethylacrylamide (DEA) performed better than LPA in terms of EOF suppression, the average extent of EOF suppression provided by these copolymers decreased with increasing polymer hydrophobicity, *i.e.*, with a higher

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percentage of DEA monomer in these random copolymers. Streaming current measurement results showed that the electrokinetic layer thickness of the adsorbed polymer layer decreased with increasing polymer hydrophobicity, which decreased the ability of the film to reduce the effective zeta potential just outside the polymer layer and hence EOF. Other polymer properties including the average molecular weight and the degree of polymerization (polymer contour length) were also determined to be critical, as they directly dictate the tendency to adsorb as well as the thickness of the adsorbed layer for polymers of this class: it was inferred from the EOF measurement data that the critical degree of polymerization needed for effective EOF suppression is ~ 15 000 [129].

3.4.2 Surface modification methods for microfluidic chips

As in CE, the surface properties of the chip substrate can have significant positive or negative effects on the performance of the devices, and surface modification of the microchannel surface is usually necessary to control EOF and DNA-surface interactions. Polymeric chips need additional attention to their surface properties due to their potentially poor compatibility with biological samples and with the organic solvents sometimes used in forming a coating [101]. Surface modification strategies for glass microchips are generally similar to those used for fused-silica capillaries. The most common strategy for surface control in a glass substrate involves the use of some form of the Hjertén protocol [125] for surface deactivation with a layer of covalently bonded polyacrylamide, and has been widely adapted for use in high-performance genotyping devices [12, 13, 83, 88], although the use of PVA-coated microchips has also been demonstrated [130]. Both of these coating methods provide excellent EOF suppression and reduce wall-analyte interactions, as reflected by the symmetric DNA peaks observed, however, the procedures used are time-consuming and labor-intensive. There is not yet a standardized protocol for applying these surface modification methods, especially in plastic chips. Dynamic coating of chip microchannels with adsorptive polymers has been also adapted as an attractive alternative, and most studies along these lines have been directed to demonstrating dynamic polymeric microchannel coatings for PCR product sizing [131–133]. HEC has been used to act as a dynamic coating for HA [134]. By using HEC as both the coating material and the sieving matrix, studies of four different alleles of the BRCA1 gene were successfully performed in glass chips, with migration times remaining stable up to 100 runs. Although this adsorbed HEC coating allowed the achievement of the necessary resolution for HA, it remains unclear whether (and unlikely that) HEC can be used in the same manner for higher performance applications such as long-read sequencing [135]. Hence, an effective dynamic coating for glass microchips that allows high-throughput, long-read sequencing has not been demonstrated thus far.

The chemistry of polymeric substrates is not as well understood as that of glass, yet many strategies have been employed to modify plastic microchannel surfaces. For example, a DMA/DEA copolymer has been used as an adsorptive coating material in a 4.5 cm polyolefin chip by ACLARA BioSciences to achieve high-speed STR sizing and DNA sequencing [14]. However, the performance of polymeric coating materials may be highly substrate-specific, and at present it is not well understood how to optimize the coating efficiency on different substrates. Chemical modification has also been employed to manipulate the chemistry of chip substrate surfaces. For example, to compensate for the hydrophobicity of the polymer substrate, sulfur trioxide gas was used to chemically modify PC [136, 137]. Sulfonation of the surface using fuming sulfuric acid produces a more hydrophilic surface. The EOF velocity above a sulfonated-PC surface is significantly lower below pH 7 than for unmodified PC. Similarly, surface modification of PMMA can be achieved by reacting the surface methyl ester groups with a monoanion of α, ω-diaminoalkanes to yield an amine-terminated PMMA surface [102, 137]. The increased hydrophilicity of modified PMMA was confirmed by the increased EOF at pH values below 8. The direction and the magnitude of EOF velocity is highly pH-dependent, due to the state of the surface amines as a function of pH. It has been demonstrated that dynamically coating a PMMA substrate with HPMC can control the EOF and allow decent dsDNA separations [138]. Along with the addition of polyhydroxyl compounds, HPMC can be used as a self-coating DNA separation matrix for this application. This system also allowed mutation detection by SSCP, but most likely would not be useful for higher resolution applications like DNA sequencing.

A gas-resin injection procedure was described by Lai *et al.* [139] for modifying the surface of PMMA for dsDNA separations. Here, hydroxyethyl methacrylate monomer (HEMA), a photoinitiator, and other additives, such as sodium dodecyl sulfate, ethylene glycol, and polyethylene glycol (PEG), were injected into the microchannel followed by applying nitrogen pressure to force the liquid out, but leaving a thin layer on the microchannel walls. The resin was then cured with UV light, and the surface was shown to be made more hydrophilic unmodified PMMA. A mixture of poly-HEMA and 10% PEG was shown to form the most hydrophilic coating as determined by contact angle measurements. Another dynamic

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coating for PMMA was used by Lin *et al.* [140] in a system designed for the separation of dsDNA: a three-layer coating consisting of a layer of PVP followed by a layer of PEO and finally a layer of 13 nm gold nanoparticles (GNPs) was effective in producing high-resolution separations of DNA by controlling EOF. Reproducibility was a problem, however, as GNPs tended to disassociate from the surface during the electrophoresis run. Better reproducibility was achieved when 13 nm GNPs were introduced into the buffer as well.

3.5 DNA labeling and detection technologies

DNA separation efficiency is limited by the capacity of the system to resolve two adjacent or partially overlapping peaks. In the case of DNA sequencing, single-base resolution is required. The resolving capacity is governed by several factors, such as signal-to-noise ratio (S/N), molecular diffusion, and dispersion (peak width), and separation effciency (peak spacing). While peak width and peak spacing can be optimized with the choice of CE conditions, wall coating, sieving matrix, and sample preparation protocols, the choice of detection methods is an important aspect of maximizing S/N. The introduction of fluorescence labeling technology for DNA enabled the use of automated detection systems for CAE [141], and facilitated multiplexing to greatly increase throughput.

3.5.1 Fluorescent energy-transfer dyes

Energy transfer (ET) dyes, invented *via* the collaborative efforts of the Mathies group and the Glazer group, exploit the fluorescence resonance energy transfer phenomenon, and greatly increased the sensitivity of ssDNA detection in multiplexed CAE systems relying on LIF detection [142]. This technology, which was successfully commercialized by Amersham Biosciences, has since become one of the standard labeling systems for high-throughput DNA sequencing and other genetic analyses, allowing all four DNA bases to be cleanly distinguished in a single "lane" with very high quantum yield. To achieve this, an improved fluorescent label was developed based an an ET "cassette" consisting of a sugar-phosphate spacer with a donor dye molecule at the 3'-end, an acceptor linked to a modified T-base at the 5'-end of the spacer, and a mixed disulfide for coupling to a thiol at the 5'-end [143]. The ET cassette has been optimized with different donor/acceptor pairs, varied spacing between the donor and the acceptor, various spacer chemistries, and an improved primer sequence [144, 145]. The major advantages of the ET dyes are that they can be almost evenly excited by laser light of a single wavelength (488 nm), which circumvents the previous inherent problem of the need for multiple excitation sources for multiplexed detection, and they produce well-separated and intense acceptor dye emission. The electrophoretic mobility shifts are minimal, and a 1.4- to 24-fold more intense signal is achieved compared to primers labeled with a single fluorophore. ET dyes have been successfully applied for multiplexing in DNA sequencing [142, 146], SNP analysis [37], STR analysis [147], and PCR product sizing [148]. However, expensive, large, and sophisticated instrumentation is generally required for sensitive LIF detection with these dyes. Another major drawback of fluorescence-based detection is that the spectral emission profiles are rather broad, hence, only a handful of dyes can be used simultaneously for multiplexing, therefore the multiplexing capability is limited. Additionally, LIF detection works best in glass microchannels: the high background signal resulting from the autofluorescence of most plastic substrate materials leads to low S/N, limiting the sensitivity of detection and hence the read length, for DNA sequencing.

3.5.2 Fluorescence lifetime dyes with near-IR detection

Alternatively, NIR DNA diagnostic methods with dye identification via fluorescence lifetime techniques have been investigated, mainly by the Soper group [149] and a few other researchers, to increase the multiplexing capability and make plastic chips more practical for high-throughput sequencing and genotyping. The fluorescence lifetime, τ_f, is an intrinsic photophysical parameter of chromophores that measures the average time difference between electronic excitation and fluorescence emission. In principle, the use of τ_f to distinguish DNA sequences can extend read length, as the lower background noise could significantly improve S/N, since only few molecules have intrinsic fluorescence in the region of interest (above 700 nm). In addition, since most capillary or chip substrate materials show no or low intrinsic fluorescence in the NIR region, the use of low-cost polymeric substrates that are problematic for conventional LIF detection (discussed above) is possible, which could further reduce the cost of sequencing and genotyping. Also, the detection instrumentation is relatively simple, since only one excitation source and detector are required. The NIR detection strategy has been proven to be useful for multiplexed, 4-channel Sanger DNA sequencing [150, 151]. In particular, in a system with a single capillary and a stationary detector, multiplexed DNA sequencing was demonstrated by Wolfrum and co-workers [152] using "pattern recognition" to distinguish τ_f for different NIR dyes, and a reported "read length" of 660 bases with a relatively low accuracy of 90.3% was achieved (researchers usually report read length with 98.5% accuracy or better). This technology

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has been further developed by Soper *et al.* [153] using multiple capillaries with a compact, time-correlated, single-photon-counting microscope for multiplexed DNA sequencing in a single-channel, long (47 cm) borofloat glass chip, and for this system a read length of 670 bases with 99.7% accuracy was reported. Novel fluorophore chemistries are under investigation to create new labeling dyes for NIR-based diagnostic applications, and which can enable DNA analysis with a higher level of multiplexing [154]. The design of the NIR labeling scheme will have important consequences on DNA sequencing performance. Currently, the major labeling dyes used for NIR-based detection are cyanine-based, and exhibit extreme hydrophobicity. This leads to potential difficulty with dye solubility and "sticking" to the microchannel surface, which may require mixed aqueous/organic solvent systems that reduce the electrophoretic separation efficiency. Hydrophobic interactions between the labeling dyes and the DNA sequencing matrix can also lead to broadened peaks and loss of DNA resolution, which ultimately limits the sequencing read length. Soper *et al.* [155] demonstrated the use of PMMA- and PC-based microchips for dsDNA analysis separations with NIR detection by electrophoresing a NIR-labeled dsDNA mixture, demonstrating a tenfold increase of speed as compared to CAE without significant loss of S/N. Since one of the challenges of using a polymeric chip substrate is the high background fluorescence, using NIR excitation can improve S/N since the level of interfering autofluorescence produced from the polymer substrate is minimized. Also, the use of a solid-state diode laser for excitation simplifies the detection instrumentation relative to conventional LIF systems which typically use an argon-ion laser [149].

3.6 Sample preparation and purification

A robust sample preparation method is critical to ensure accurate amplification of the target DNA and the purity of the samples, and has been shown to be critical for obtaining high resolution in different genotyping strategies [34, 156–158]. Novel sample preparation and purification methods have been developed to enhance sample quality and facilitate automation. In addition, low-volume sample preparation methods have been shown to significantly reduce reagent consumption. The ability to deal with low sample volumes is particularly important when working with forensic samples, which because of DNA degradation often have a low concentration of full-length template sequence of interest [158].

A conventional, multiplexed Sanger cycle sequencing reaction requires relatively large-scale samples (μL), off-line PCR, and sample pooling and purification, potentially creating a bottleneck in upstream sample preparation as well as in downstream, automated electrophoretic separations. To address this limitation, a novel Sanger sequencing reactor has been demonstrated by Soper *et al.* [159] using a solid-phase nanoreactor directly coupled to CE, which promises reduced reagent consumption and streamlining of operation. Capillary-based, low-volume sample preparation strategies that involve nanoliter-scale reactions inside bare fused-silica capillaries have also been demonstrated by the Yeung and Jovanovich groups [160–162]. These on-line, low-volume sample preparation methods create sequencing samples that give comparable peak efficiency and S/N to macroscale sample preparation protocols, and hold promise for integrated CAE and/or chip applications. A simple, automated strategy to couple nanoreactors to multiplexed CAE or chips will allow the exciting potential of these systems from to be realized. One proposed coupling method involves the use of a microfabricated hydrid device coupled to CAE for high-throughput DNA sequencing, allowing the introduction of low-volume samples through microfabricated injectors [163].

The need for sample purification prior to CE or microchip electrophoresis analysis presents further challenges for creating a fully integrated system. Several impurities (template DNA, chloride ions, buffer components, and unincorporated nucleotides) which appear in the product matrix of the Sanger reaction are known to have deleterious effects on obtaining high S/N in DNA sequencing electropherograms. Ethanol precipitation or solid-phase extraction (SPE) are usually employed for sample purification, however, this is time-consuming and the results are not fully satisfactory [157]. Several novel methods have been investigated to enhance the product quality and hence reduce background signal due to impurities. A detailed protocol has been established to remove DNA template using an LPA-treated poly(ether sulfone) ultrafiltration membrane, while salt, buffer components, and nucleotides are removed with two prewashed SPE spin columns prior to injection [156]. A tenfold improvement in S/N was observed after the cleanup due to the reduced impurities and the increased sample loading, allowing a substantially longer read length [157]. However, this technology still requires off-line operation, which could create a bottleneck in the operation, and also is probably too expensive for genome centers to use. Yeung *et al.* [164] developed a more practical capillary zone electrophoresis-based system to extract DNA extension fragments, which are then directly injected into a manually coupled gel-filled capillary used for DNA sequencing. This system demonstrated the feasibility of nano-volume DNA analysis, while at the same time providing an important proof-of-concept study for an integrated DNA purification

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and analysis system. This automated design laid the foundation for integrated microchip systems which perform these tasks.

In an integrated microfluidic device, new sample preparation and purification strategies are being developed that allow the extraction of high-quality DNA from a complex sample, such as a white blood cell, from a much-reduced sample volume relative to standard methods, with effective coupling to an electrophoretic size- and/or conformation-based separation channel. Currently, most sample preparation protocols involve off-chip operations performed on the microliter scale. Recently, however, Soper *et al.* [165] demonstrated a capillary nanoreactor coupled to microchips for the preparation of DNA sequencing ladders and PCR products. The design consists of fused-silica capillary tubes with EOF as the driving force for fluid transport, thermostated in fast thermal cyclers for PCR and Sanger sequencing reactions. The capillaries are coupled to PMMA microchips *via* capillary connectors. The hybrid device allows the integration of automated sample preparation and analysis without a loss of efficiency.

As mentioned above, current DNA purification processes typically rely on SPE of DNA *via* hydrogen bonding or electrostatic interaction to μm-scale beads. A significant step toward miniaturized and integrated sample purification has been demonstrated by the Landers group [166] utilizing a low-volume flow cell for SPE of DNA from cell lysate, for genetic mutation detection applications. Microchip-based purification devices have been further developed by Landers [167] using sol-gel/bead composites, with good DNA extraction efficiency and reduced analysis time. Flow conditions through these systems can be optimized at various pHs to enhance DNA extraction efficiency. At the optimal load buffer pH (6.1), a flow rate as high as 250 μL/h is applied to purify DNA from a blood sample in less than 15 min with a microfabricated SPE device consisting of silica sol-gel [168]. Solid-phase reversible immobilization (SPRI) is a related approach that has been utilized by the Soper group [169] for the on-chip purification of dye-labeled DNA sequencing fragments. A novel SPRI strategy has been developed using photoactivated PC in a microchip to increase the loading level of DNA. An immobilization bed of PC is produced by exposing a posted microchannel to UV radiation, which induces a surface photo-oxidation reaction. The resulting surface-bound carboxylate groups show strong affinity for DNA fragments suspended in an immobilization buffer (Tris-EDTA-glycerol (TEG)/ethanol). After releasing the DNA from the surface by incubation with deionized water, followed by electrokinetic injection into a separation channel, a read length of 620 bases with a baso-calling accuracy of 98.9% was achieved. At present, this method yields lower plate numbers for DNA peaks, as compared to direction injection of off-line purified samples, but perhaps can be optimized [169].

The use of both SPE and SPRI presents challenges in the effective packing and localization of the extraction materials into small chip reservoirs. Other on-chip purification strategies are being investigated to address this problem, which also hold promise for an integrated sample purifcation and analysis device. Hydrogel plugs functionalized with DNA inside a microchannel have been used to selectively capture oligonucleotides, *via* DNA hybridization, for the preconcentration of DNA samples [170]. In particular, Mathies *et al.* demonstrated an integrated sample purification device by utilizing gel-immobilized DNA capture probes attached to a replaceable acrylamide-methacryl-DNA copolymer, to selectively trap Sanger reaction DNA extension fragments for sequencing. A schematic diagram of this approach is presented in Fig. 3. Sample preconcentration and desalting was accomplished by a two-step, electrophoretically driven capture and washing procedure. A ~ 200-fold increase of volumetric concentration was achieved in only 120 s. The direct injection of the preconcentrated sample demonstrated that the process could be automated in the integrated device [171].

Another approach that involves the use of water-soluble poly(acrylamide-*co*-alkylacrylamides) for electrophoretic DNA purification (from proteins) has been demonstrated in our laboratory [172]. This idea exploits the proteins' hydrophobic interaction with the hydrophobically modified acrylamide copolymers to selectively remove proteins from DNA *via* microchannel electrophoresis. DNA, on the other hand, does not "stick" to the polymer network. A series of *N*-alkyl acrylamide co-monomers with varying alkyl chain lengths (C4, C6, C8) and also an *N,N*-dialkyl group (C6, 6) were synthesized *via* reactions between acryloyl chloride and the respective alkyl amines. Copolymers were synthesized using an aqueous "micellar" polymerization technique, which involves dissolving acrylamide in the aqueous phase while hydrophobic monomers are solubilized in SDS micelles. The resulting copolymers, with molecular masses ranging from 1.5 to 4.3×10^6 g/mol, comprising up to 4 mol% of a hydrophobic subunit (as verified by ^{1}H NMR), were used to adsorb the proteins. CE analysis of bovine serum albumin (BSA) and β-lactoglobulin migration in these matrices revealed that the octylacrylamide and dihexylacrylamide copolymers show the most significant extent of protein adsorption, while butyl acrylamides show no noteworthy adsorption trend. All copolymer matrices studied allowed the passage of a dsDNA digest, and displayed some DNA

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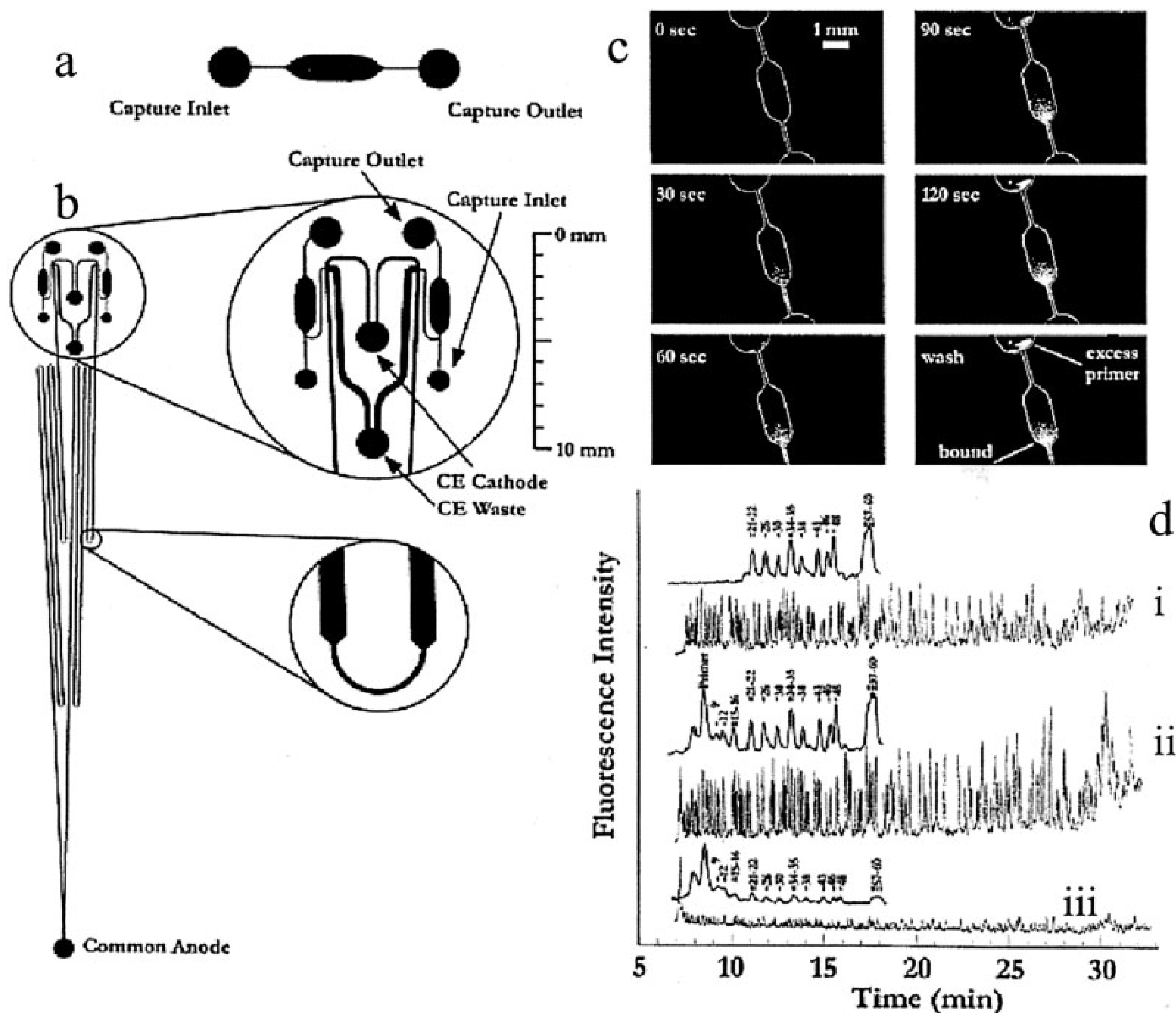

Figure 3. (a) Design of a capture chamber for DNA template binding optimization; (b) schematic of the CE DNA sequencing channel integrated with a capture chamber; (c) epifluorescence images showing the progress of a typical oligonucleotide capture operation in which the product is concentrated exclusively in the inlet (bottom side) taper region; (d) (i) On-chip oligonucleotide capture-purified M 13mp 18 four-color sequencing sample, (ii) An aliquot of the same sample was precipitated with ethanol, resuspended in 50% formamide in doubly deionized (DI) H_2O, (iii) the same unpurified sample was directly analyzed in parallel with the precipitated sample. Reprinted from [171], with permission.

sieving ability at 0.5% w/w in Tris-TAPS-EDTA (TTE) buffer. These water-soluble matrices allow easy and convenient implementation of integrated microfluidic devices that incorporate streamlined DNA purification and analysis. Alternative purification methods using analyte focusing have been proposed and demonstrated by the Locascio and the ACLARA BioSciences groups. These approaches modulate analyte electrophoretic migration spatially through the use of a temperature gradient [173] or with buffer ion mobility [174], with demonstrations so far limited to simple (nonbiological) samples.

3.7 DNA separation matrices

The development of DNA separation matrices remains an important endeavor, as the properties of the sieving polymers directly dictate the separation resolution and migration behavior of DNA molecules, as well as the difficulty or ease of microchannel loading of the matrix [175–177]. A number of different polymers, other than the well-studied polyacrylamide and acrylamide derivatives, have recently been used for genotyping or dsDNA fragment sizing including methylcellulose (MC) [178], hydroxyal-

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kylcelluloses [134, 179], poly-*N*-acryloyl aminopropanol (AAP) [180–182], and PVA and its copolymers [183]. For DNA sequencing, in which single-base resolution is required over a wider size-range, the search for an ideal polymer matrix for chip-based separations continues. Current research has been relying mainly on polyacrylamide and other polymers and copolymers of acrylamide derivatives. Since the development of replaceable, ultrahigh-molar-mass LPA matrices by Karger *et al.* [67–69, 184], LPA has been the matrix of choice for high-throughput DNA sequencing. Ultrahigh-molar-mass LPA solutions can give excellent sequencing performance, but is perhaps not an ideal sequencing matrix, especially for chip-based applications, for three reasons: (i) its high viscosity, (ii) its lability to hydrolysis at high pH, and (iii) its lack of an intrinsic wall-coating ability. It is desirable to develop a low-cost, self-coating, low-viscosity, yet highly effective separation matrix for long-read sequencing in microchip electrophoresis devices. Different polymer types might be ideal for uses in glass *vs*. plastic chips. The properties of a number of other water-soluble polymeric materials for DNA sequencing by microchannel electrophoresis have been studied intensively, and the development of polymeric DNA separation matrices of CE and chip electrophoresis has recently been reviewed [185–187]. Here, we discuss mainly the recent reports of novel thermo-responsive [188] and self-coating [122, 126, 128, 189, 190] polymeric matrices, and a few non-conventional separation matrices that have been demonstrated for DNA analyses.

3.7.1 Linear polyacrylamide

Although numerous types of water-soluble, high-molar-mass polymers have been investigated for sequencing applications, LPA is still the most widely accepted high-performance DNA sequencing matrix. LPA is also a highly useful medium for other genetic analyses, being an effective sieving matrix for *(e.g.)* mutation detection by tandem SSCP/HA [33, 38]. LPA possesses a few very desirable properties, particularly its very high hydrophilicity and excellent DNA-separating ability. High-molar-mass LPA, which is most effective in DNA sequencing, is synthesized by inverse emulsion polymerization. A few novel concepts have been proposed to further enhance the performance of LPA matrices.

3.7.1.1 Sparsely cross-linked "nanogels"

A new concept has been introduced to provide additional network stabilization for high-molar-mass LPA by sparse chemical crosslinking of discrete, subcolloidal polymer domains, as illustrated in Fig. 4. This type of material is created by incorporating a very low percentage of cross-linker ($< 10^{-4}$ mol%) in high-molar-mass LPA made by inverse emulsion polymerization.

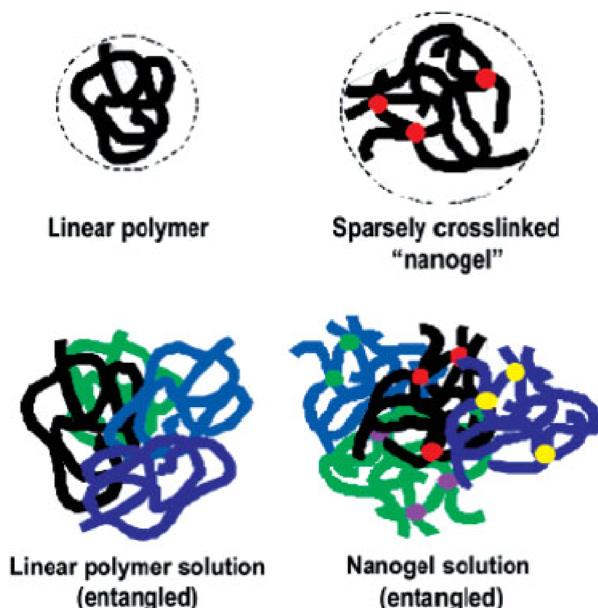

Figure 4. Schematic representation of nanogels (right) and linear polymer chains (left). Reprinted from [191], with permission.

Since cross-linking is localized, these matrices flow. Nanogels have an average M_w of 10–20 $\times$ 10^6 g/mol, and $R_g \sim 220$–240 nm. Physical polymer network stabilization using sparsely cross-linked "nanogels" gives enhanced ssDNA separation selectivity and hence extended DNA sequencing read length in CAE [191]: the initial studies showed a 10% improvement of DNA sequencing read length over a LPA matrix of similar molar mass and extent of entanglement. In addition, "nanogel" matrices are optimally used at a lower concentration, and require ~ 30% less polymer than LPA with the same molar mass, hence offering relatively easy microchannel loading. Further optimization of the "nanogels" with respect to the cross-linker concentration shows that "nanogel" matrices with 4×10^{-4} mol% cross-linker can provide an 18% longer read length than a matched LPA counterpart. This type of sparsely cross-linked "nanogel" network has also been applied successfully in a microfabricated sequencing device, yielding a DNA sequencing read length of around 500 bases at 98.5% accuracy in 25 min [192].

3.7.1.2 Ultrahigh-molar-mass LPA made by plasma-induced polymerization

A novel approach, using plasma-induced polymerization (PIP), has also been developed to synthesize LPA for DNA separations [193]. This approach aims at producing

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ultrahigh-molar-mass polyacrylamide, which when dissolved in dilute aqueous solution, possesses ultrahigh viscosity and provides high-resolution DNA sequencing separations. PIP is a nonconventional polymerization method, which involves the generation of a cold plasma to produce the initiating species, which in turn generate "living macroradicals" in the post-polymerization period. This method was used to produce polymer products with ultrahigh molecular mass and reportedly low polydispersity (though this property can be very difficult or impossible to accurately assess in such high-molar-mass polymers [194]). These properties are believed to be ideal for high-resolution electrophoretic DNA separations, particularly for sequencing. Even dilute solutions of this polymer, produced by PIP, have very high viscosity, and were shown to provide very effective DNA sieving, as shown in the separation of ssDNA markers in the same study, as illustrated in Fig. 5. However, the use of this technology does not address the practical difficulties associated with using highly viscous sieving solutions in miniaturized systems. In addition, it is difficult to accurately analyze the physical properties of these polymers, in particular to measure their average molecular weight and size distribution [194], which have found to be important parameters to control for DNA analysis [69, 176].

3.7.2 Thermo-responsive polymer matrices

The use of thermo-responsive polymeric materials as DNA sieving matrices for CE was pioneered by Sassi and co-workers in 1996 [195, 196]. Since then, a number of research groups have been involved in developing thermo-responsive polymers for dsDNA analysis [197, 198] or single-color ssDNA separation [199], while the design of these "switchable-viscosity" networks for 4-color DNA sequencing has been mainly pursued by Barron *et al.* [188, 200, 201]. Ideally, these matrices allow a decoupling of the capillary loading and DNA sieving performance, and if developed further, may be one of the key technological developments that enable the practical use of microfabricated devices for high-throughput sequencing and genotyping [188]. By hamessing the dramatic viscosity change associated with the volume-phase transition of thermo-responsive polymer networks, rapid loading of sieving matrices into capillary or chip microchannels can be accomplished with a moderate applied pressure, and effective DNA sieving can be achieved upon the restoration of the entangled state of the polymers. The use of these matrices could address the problem of the low pressure tolerances of most microchips (about 200 psi for a glass chip, and as low as 50 psi for chips made of polymeric materials). Most thermo-responsive polymeric matrices have only been demonstrated for dsDNA separations; this is true of matrices based on poly-*N*-isopropylacrylamide (pNIPA) [195] and hydroxypropylcellulose (HPC) [179, 195]. However, a thermo-responsive matrix based on copolymers of DEA and DMA has been successfully formulated, characterized, and applied for DNA sequencing by CE [200]. In this work, a random copolymer network composed of 42% w/w DEA and 58% w/w DMA delivered 575 bases in 94 min with a base-calling accuracy of 98.5% [188]. The thermally controlled "viscosity switch" of this matrix allows easy capillary loading, by inducing a two-order of magnitude drop of viscosity when heated above the lower critical solution temperature (LCST), while polymers are entangled and the viscosity is high under DNA separation

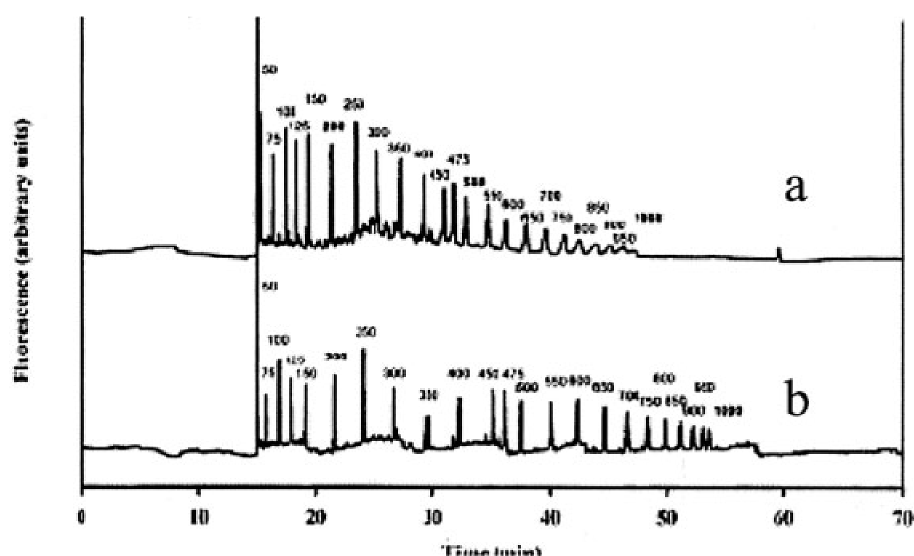

Figure 5. Electropherograms of a Cy5 ssDNA marker using 3% solutions consisting of LPA synthesized by (a) conventional solution polymerization, and (b) nonconventional PIP. Reprinted from [193], with permission.

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conditions. This is a "thermo-thinning" matrix, with high viscosity at low temperature and lower viscosity at higher temperature, above the LCST. Polymer blends of various DMA/DEA copolymers, with different LCSTs, also exhibit an interesting "dynamic porosity" property, in which the separation selectivity of dsDNA of various sizes showed different temperature-dependencies [202].

On the other hand, "thermo-thickening" polymer networks exhibit an upper critical solution temperature (UCST) at which an expansion of coil volume occurs, accompanied by thermo-association of polymer chains and a dramatic increase in viscosity. The thermally actuated gelation can allow easy microchannel loading at or near room temperature, followed by heating to the sequencing temperature (usually 45–65°C) to form a physically cross-linked "gel" for effective DNA sieving. A number of "thermo-thickening" polymeric sieving matrices have been developed based on polymers that exhibit thermo-associative behavior, with novel copolymer architectures, such as poly-*N*-isopropylacrylamide-graft-polyethylene oxide (pNIPA-*g*-pEO) [203, 204], poly-*N*-isopropylacrylamide-graft-polyacrylamide (pNIPA-*g*-LPA) [199], and polyethylene oxide-polypropylene oxide block copolymers (pEO-pPO-pEO) [205] being investigated. These polymers utilize the self-associating properties of the hydrophobic chain parts, which serve as physical cross-linking points, to form extended polymer networks when heated above the transition temperature. The above-mentioned three classes of "thermo-thickening" matrices have shown the ability to give high-resolution dsDNA separations. Very recently, thermo-gelling polymeric matrices based on copolymers of two different *N*-alkoxyalkylacrylamide monomers have been applied for 4-color DNA sequencing by CE [201]. A read length of around 600 bases, with base-calling accuracy of 98.5% was achieved with a thermo-gelling copolymer ($M_W \sim 2.5$ MDa) composed of 90% w/w *N*-methoxyethylacrylamide (NMEA) and 10% w/w *N*-ethoxyethylacrylamide (NEEA). The temperature-dependent viscosities of several copolymers are shown in Fig. 6. With further optimization, it is believed that the DNA sequencing performance of *N*-alkoxyalkylacrylamide-based polymers will be improved, and that this class of polymers is a promising candidate for high-performance microchip-based electrophoretic DNA sequencing.

A major drawback of both types of thermo-responsive polymeric materials discussed above (both thermo-thinning and thermo-thickening) is that the inclusion of hydrophobic moieties in the polymer structure is required to achieve thermal sensitivity of polymer phase behavior, and DNA separation efficiency is adversely affected by matrix hydrophobicity because it weakens the entangled polymer network [175]. While the incorporation of a larger portion of hydrophobic comonomers often leads to a more significant "viscosity switch", the DNA separation efficiency is usually compromised in this case. Hence, the chemical and physical structures of thermo-responsive polymer networks must be finely tuned to optimized their performance for DNA sequencing on chips and they may never yield read lengths quite as long as LPA does.

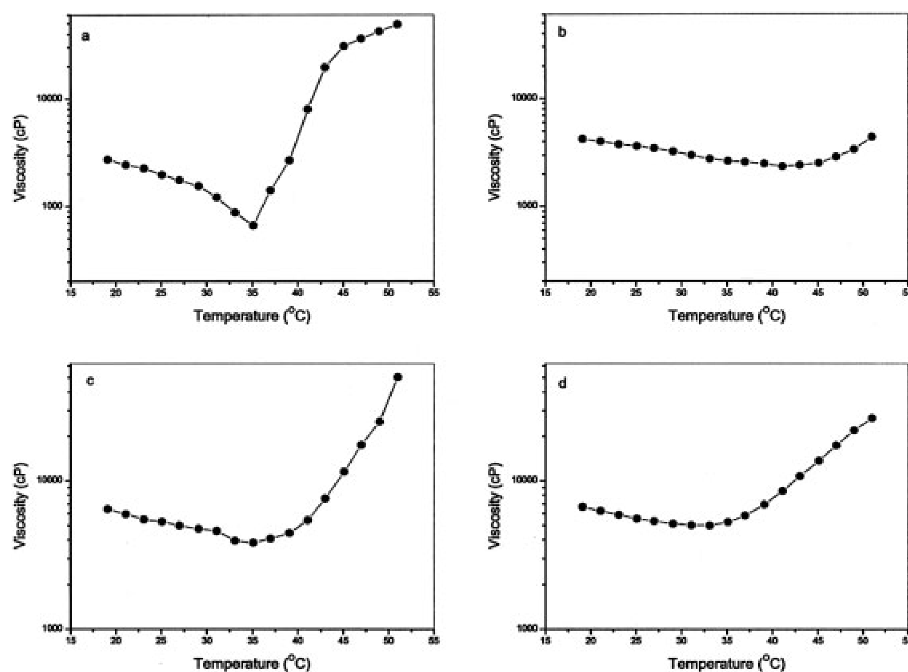

Figure 6. Temperature-dependent viscosities of (a) pNEEA, (b) pNMEA, (c) a copolymer consisting of 25% NEEA and 75% NMEA w/w, and (d) a copolymer consisting of 10% NEEA and 90% NMEA w/w in 0.5X TTE/7 M urea solution at 7% w/v concentration. Experiments were performed with temperature control in a cone-and-plate fixture (diameter 25 mm; angle 2°) at a heating rate of ~ 2°C/min. Applied shear rate was 1 s^{-1}. Reprinted from [201], with permission.

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3.7.3 "Self-coating" (adsorptive) polymer matrices

Another major portion of the cost of genome sequencing is attributed to the purchase of high-quality fused-silica capillaries internally coated with polymers to eliminate EOF and minimize analyte adsorption. Presently, arrays of 96 capillaries are most commonly used. While DNA molecules tend not to adsorb to the silica surface at pH 7–8 due to electrostatic repulsion between the negatively charged wall and the anionic phosphodiester backbone of DNA, ssDNA has a substantially more hydrophobic character than dsDNA and can interact with the silica wall; thus, in DNA sequencing, coated capillaries are always used. The production cost of the labor-intensive preparation of a covalently coated capillary arrays is rather high, and the use of self-coating matrices is an attractive solution to help drive down the cost of sequencing and genotyping projects. Poly-*N,N*-dimethylacrylamide (pDMA) and its derivatives [206] and poly(ethylene oxide) (pEO) [128] are the most common self-coating matrices that have been demonstrated for DNA sequencing. Although pDMA reduce EOF significantly [129], its somewhat hydrophobic character limits the DNA sequencing to about 600 bases [206]. Recently, a novel group of copolymers, poly(DMA-*co*-allyl glycidyl ether), were used by Chiari *et al.* [207] as a self-coating polymer matrix for DNA fragment sizing. These novel copolymer matrices were found to be very stable at high pH, high temperature, and in the presence of a denaturant, which makes them potentially useful for different genotyping approaches. To date, however, DNA sequencing has not yet been demonstrated with these new matrices. A read length of 1000 bases was achieved with the use of high-molar-mass pEO as a self-coating matrix for DNA sequencing, however, this required an extended analysis time (7 h) and a long separation distance due to the poorer DNA separation performance of the intrinsically very flexible pEO networks under high electric fields [208]. Poly-*N*-hydroxyethylacrylamide (pHEA or polyDuramide™) is another polymer that has been developed as a uniquely hydrophilic self-coating matrix for DNA sequencing [122]. Absorbed pHEA coatings provide more than 2 orders of magnitude reduction of EOF velocity, and as entangled polymer networks yielded a DNA sequencing read length of $>$ 700 bases with a base-calling accuracy of 98.5%. In addition, pHEA has been shown to be an effective wall-coating material for high-resolution genetic mutation analysis by tandem SSCP/HA [33, 34].

3.7.4 Interpenetrating polymer networks

Interpenetrating networks (IPNs) of various polymers have been investigated by the Chu group as a way to combine the desirable properties of different polymers for electrophoresis, and as an alternative to the use of copolymers. The constituent polymers are usually non-miscible, making the use of direct polymer blends impractical. The use of IPNs reportedly enables the mixing of normally immiscible component polymers without phase separation, hence creating a homogenous sieving solution which functions to give high-resolution DNA separations. For example, an IPN of polyvinylpyrrolidone (PVP) and LPA has been synthesized by polymerizing acrylamide monomer directly in a PVP/buffer solution, and subsequently used for dsDNA separation [209]. PVP possesses excellent dynamic coating ability, however, the intrinsic viscosity of PVP solutions is too low to provide effective size-based separation of DNA. Hence, it was desirable to incorporate another polymer to improve the sieving performance.

Interestingly, the dsDNA separation performance of the PVP/LPA IPN was far better than PVP alone, LPA alone, or a PVP/LPA blend. In another, similar study, an IPN of PVP and pDMA was synthesized by polymerizing DMA monomer directly in a PVP/buffer solution, and utilized for dsDNA separation [210]. This study was aimed at applying the same concept using less compatible polymers than the former system (PVP is less miscible with pDMA than with LPA). Again, the dsDNA separation resolution in the PVP/pDMA IPN was superior to that obtained with PVP alone and with a blend of PVP and pDMA. The authors hypothesized that the superior performance of the IPNs could be attributed to an increase in the number of entanglements by the more extended polymer chains, which could be inferred from the high apparent viscosity of the IPNs.

3.7.5 Nonconventional DNA sieving matrices

Several other concepts have been proposed and demonstrated as alternatives to using high-molar-mass, highly viscous polymer networks. For example, the development of monomeric, nonionic surfactants, consisting of *n*-alkyl polyoxyethylene ethers, as "dynamic polymers" has been demonstrated [211]. These monomeric, nonionic surfactants reportedly can serve as "reversible gels" by self-assembly and allow effective dsDNA size-separation and ssDNA separation for fragments up to 600 bases long by CE using a BigDye primer G-labeled M13(-21) sequencing sample (single-color). However, the use of these self-assembling surfactants for four-color sequencing has not yet been explored, so their true potential is unknown. This class of materials is reported to offer advantageous properties such as ease of preparation, low viscosity, and EOF suppression ability. The "porosity" of the self-assembled surfactant network

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was said to be easily controlled with the choice of monomer concentration, denaturant, and temperature. A related family of self-assembling hydrogels consisting of aqueous solutions of PEG, end-capped with perfluorocarbon groups, has been applied for dsDNA fragment sizing [212]. The aggregation of hydrophobic fluorocarbon cores acts as physical cross-linking points, so that physical gels form at relatively low surfactant concentrations (as low as 2% w/w) and can provide high-resolution dsDNA sieving. However, these self-assembled hydrogel networks are apparently only applicable for the separation of small dsDNA (up to ~ 400 bp). Larger DNA molecules could not be resolved due to the weakness of the self-assembled surfactant network.

Another alternative to the use of polymeric DNA sieving matrices was recently published by Baba and coworkers [213] and involves the use of core-shell type globular nanoparticles (nanospheres). This novel type of DNA sieving nanoparticles are prepared by the multimolecular micellization and subsequent core polymerization of block copolymers of PEG with poly(lactic acid) (PLA) possessing a methacryloyl (MA) group at the PLA chain end, resulting in the synthesis of a macromolecule of the chemical structure PEG_m-β-PLA_n-MA. When dissolved in aqueous solution, the hydrophobic PLA segments form a spherical core, which is surrounded by a corona of tethered, flexible PEG chains. The MA groups can be polymerized further to form stable core-shell type nanospheres with a diameter of 30 nm. These nanoparticles reportedly form a stable, close-packed structure in microchannels at concentrations as low as 1% w/v, and provide effective dsDNA separation for fragments ranging in size from 100 bp to 15 kbp within 100 s of electrophoresis. The wide range of separable DNA fragment sizes is attributed to a delay of onset of reptation in these matrices, as inferred from mobility *versus* DNA fragment size data and fluorescence microscopy. Given the phenomenal separation efficiency and range which was reported, it will be interesting to see if this nanogel-based matrix can separate a ssDNA size ladder with single-base resolution as required for DNA sequencing.

Another intriguing type of nonconventional DNA sieving matrix was demonstrated recently by the Viovy group [214, 215]: a magnetically self-assembled DNA "obstacle course". In this work, the reversible formation of a self-organized, quasi-regular array of columns in fluid trapped between two microscope slides occurs when a constant, homogeneous magnetic field is applied to a suspension of superparamagnetic particles confined in a thin (μm-scale) gap perpendicular to the field. The reversibility is attributed to the dissembly of the arrays to a liquid suspension upon field switch-off, which translates to the possibility applying them as low-viscosity DNA sieving solutions. The fact that the spacing (pore size) of these columns could reportedly be tuned with the field, with good reproducibility, could allow the tailoring of matrix properties for DNA separation over different size ranges. As shown in Fig. 7, the electrophoresis of a zone of intact λ DNA (48.5 kbp) has been demonstrated, shown with a low theoretical number of plates for that zone of 7000 [215]. In other work, these self-assembling matrices of magnetic bead columns were used in a microfluidic device with a computer-piloted flow control system and injection for the electrophoretic separation of long DNA [214]. Rapid separation of λ-phage (48.5 kbp), 2λ-DNA (97 kbp), and bacteriophage T4 DNA (168.9 kbp), with separation resolution greater than 2 between λ, and T4, was achieved in less than 150 s. The experimental findings were compared with theory, which was developed using an exact solvable lattice Monte Carlo model. It was asserted that the mean velocity of DNA migration scales linearly with the applied electric field, while band-broadening scales with the inverse of the field, resulting in the fteld-independent resolution for intermediate fields; there was strong agreement between experimental results and theoretical predictions. This invention represents a novel concept in "matrix-based" DNA separation with results not easily achievable with conventional DNA sieving matrices based on high-molar-mass polymer solutions. However, this type of separation medium may not be ap-

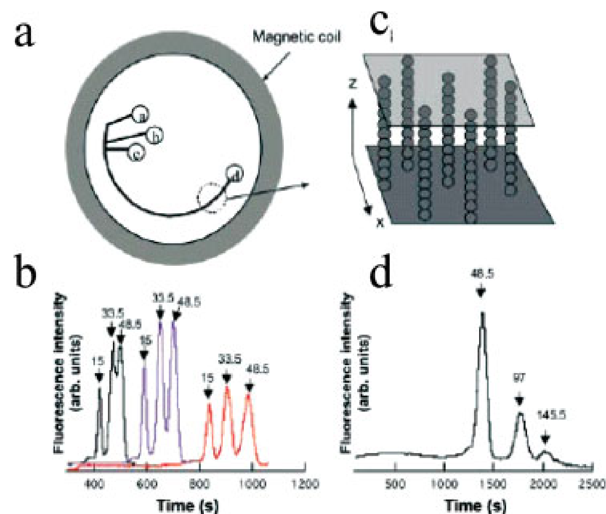

Figure 7. (a) Schematic of the microchannel and magnetic coil; (b) columnar structure formed by a suspension of superparamagnetic particles; (c) fluorescence intensity at 10 mm from the injection zone *versus* time. Separation of a mixture of 1-phage DNA and 1-DNA digested with *XhoI* (the numbers refer to the size of the DNA fragments in kbp); (d) separation of 1-phage DNA concatemers. Reprinted from [215], with permission.

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plicable to DNA sequencing, given that all demonstrations so far have shown the separation of extremely large dsDNA molecules.

3.8 Integration of functions in microfluidic devices

The ultimate goal of miniaturization is to perform multiple functions on a single platform, creating a true μTAS. A number of proof-of-concept μTASs have been developed and demonstrated for simple chemical analyses [216, 217]; however, only a handful of integrated genotyping devices have been demonstrated due to the sample complexity and the separation resolution required. Although the microfluidic technologies discussed above occasionally have shown superior separation performance or advantageous properties (such as low cost and high sensitivity) compared to conventional large-scale sequencing and genotyping instrumentation, the required ancillary steps such as sample preparation have mostly been done off-line and are usually several orders of magnitude larger in volumetric scale; hence, the true power of miniaturization has yet to be seen. The development of integrated genetic analysis devices has been mainly pursued by the Mathies group, the Quake group, the Ramsey group, and the Landers group.

The general approach to device integration involves the combination of different unit operations, such as mixing, energy transfer, biocatalytic reaction, product extraction or waste removal, separations and detection, into micro- or even nanoscale volumes, demanding extraordinarily precise control of process parameters both spatially and temporally for success. It is not a trivial issue to integrate multiple processes into a single chip, and thus far only the integration of PCR and electrophoretic separation has become routine on a single device [44, 218–221]. An example of an integrated PCR/dsDNA separation chip is shown in Fig. 8. The integration of multiple operations on a single platform for genetic analyses is still in a relatively early stage of research.

The achievement of fully integrated and automated DNA analysis devices, especially for high-throughput DNA sequencing and genotyping, will still require significant innovations in integration strategy, and important technical issues such as the interfacing of different processes will have to be carefully addressed.

Chips that integrate and streamline the entire sequencing process including cell sorting, colony picking, template preparation, the cycle-sequencing reaction, and electrophoretic separation can be envisioned [76, 79, 100, 120, 168, 171, 222]. Current state-of-the-art technologies

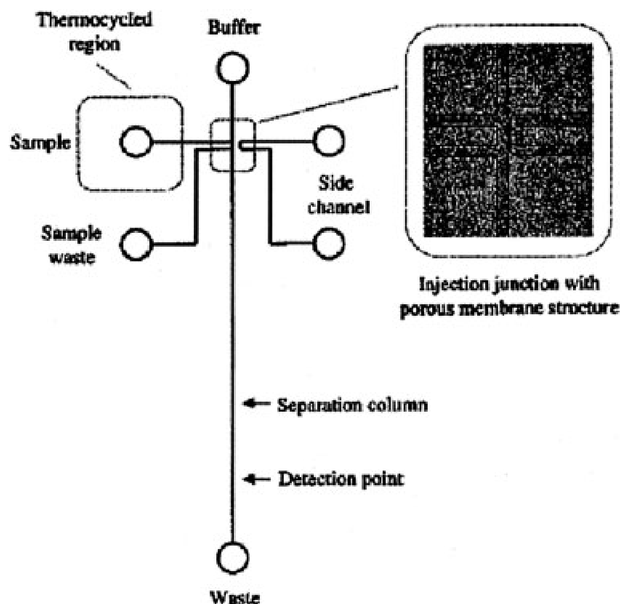

Figure 8. Microchannel design used for integrated rapid PCR analysis on-chip. Reprinted from [218], with permission.

allow the combination of unit operations at different stages along the line, such as integrated chip-based sequencing reaction/PCR and electrophoretic separation of DNA [120, 220, 221, 223], integrated sample purification and sequencing analysis [171], and integrated front-end processes, such as cell sorting and manipulation [77, 222]. An inherent challenge in combining these steps is the very different process conditions and kinetics involved.

Significant breakthroughs in the development of integrated microfabricated sequencing and genotyping devices have been realized in different contexts. For example, large-scale integration has been shown for microscale materials manipulation and integrated PCR and DNA fragment analysis [76, 80]. On-chip detection systems, such as an integrated photodiode detector [224], will allow the shrinking of the detection systems and could make integrated microscale detectors practical. The inventions of novel temperature control methods such as infrared-mediated thermal control [225, 226] allow precise control of temperature in different regions of the microfabricated devices, which will be important as the various processes that need to be integrated have very different temperature requirements.

4 Summary

Recent progress in developing different aspects of miniaturized sequencing and genotyping systems is beginning to clarify the challenges we face in creating the next-

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generation biomedical technology. While a number of innovative approaches are under development that might have a major impact on the future of genetic analyses, such as single-molecule sequencing [227] and DNA separation by entropic trapping on microfluidic devices [228, 229], a practical high-throughput device for sequencing and genotyping based on these new technologies will most likely not be realized for at least 5–10 years. On the other hand, it is possible that high-throughput chip-based sequencers relying on electrophoretic DNA separation could be commercialized in 2–3 years. Because of the numerous advantages one gains with the use of miniaturized systems, the research focus of many groups will likely continue toward the goal of creating integrated μTAS for genetic analyses. In addition to the more "traditional" routine of analyzing DNA molecules which are separated by electrophoresis in a sieving medium, followed by fluorescence detection, other approaches, in particular the use of on-chip electrochemical detection [230–232], and matrix-free methods such as end-labeled free solution electrophoresis (ELFSE) [233] are also under active development. These new research initiatives will probably be important for the development of practical microfabricated genetic analysis devices. With the use of electrochemical detection, the "clumsy" LIF detection system could also be miniaturized and integrated into a single platform. On the other hand, ELFSE or other matrix-free methods will eliminate the need of using highly viscous polymer sieving solutions, making the use of chip devices more feasible.

Meanwhile, there are several unmet challenges to be faced in the development of a practical integrated chip device for sequencing and genotyping, both scientific and nonscientific. In particular, while most existing technologies show excellent performance with model samples, the analysis of real-world samples is much more complicated, as analytes are usually present in low concentration within a complex sample matrix. As we discussed in the beginning of this article, we believe there are actually no prohibitive technological limitations preventing the creation of an integrated μTAS chip. We therefore postulate that there are some nonscientific obstacles which have slowed the introduction of this technology. Firstly, given the highly multidisciplinary nature of this endeavor, we need a driving force to foster collaboration between researchers with different expertise, to promote rapid technological innovation. Secondly, it may take a while for practitioners to adopt the new technology with the need for new capital investment, as well as the time and cost to train laboratory personnel, which may seem at first to outweigh the advantages of a reduction in operating costs and an increase in throughput. Finally, the cost for biotechnology companies to invest in developing and commercializing this new technology, which most likely will supplant the CAE instruments they are currently marketing (which were themselves developed at great expense) should not be underestimated, and will undoubtedly affect managerial decisions as to when to explore and create this new market.

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