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Integrated System for Rapid PCR-Based DNA Analysis in Microfluidic Devices

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An integrated system for rapid PCR-based analysis on a microchip has been demonstrated. The system couples a compact thermal cycling assembly based on dual Peltier thermoelectric elements with a microchip gel electrophoresis platform. This configuration allows fast (∼1 min/cycle) and efficient DNA amplification on-chip followed by electrophoretic sizing and detection on the same chip. An on-chip DNA concentration technique has been incorporated into the system to further reduce analysis time by decreasing the number of thermal cycles required. The concentration injection scheme enables detection of PCR products after performing as few as 10 thermal cycles, with a total analysis time of less than 20 min. The starting template copy number was less than 15 per injection volume.

The development of Lab-on-a Chip technology is directed toward virtually hands-free chemical and biochemical analysis on microfabricated devices. The concept is based on miniaturization and integration of liquid handling, sample processing, and analysis on a single device. Analysis methods with improved speed and automation and reduced reagent consumption are central to fulfilling the increasing demands of biological research and applications. There are two major problems to be addressed in regard to the implementation of polymerase chain reaction (PCR)-based DNA analysis in microfabricated devices: (1) increasing thermal cycling rates and efficiency of PCR amplification and (2) incorporating DNA-compatible microreactors into the microchip format to perform an integrated analysis.

Miniature devices for PCR-based analysis of DNA are currently being developed in a number of laboratories. A microfabricated silicon heater mated to a glass microchip for electrophoretic analysis of PCR products has been reported.[1] Recently, we have demonstrated an integrated device that accomplishes cell lysis, multiplex PCR, and capillary electrophoretic (CE) size analysis on monolithic microchips using bacterial samples.[2] Approaches to increasing the throughput and functionality of these devices for genetic analysis have been undertaken using multiple reaction wells for parallel DNA amplifications[3] and the implementation of on-chip concentration of DNA fragments prior to subsequent electrophoretic separation.[4] Integrated PCR-CE microchips have also been used for the analysis of simple sequence repeat polymorphisms in mammalian genomes.[5] Multiplex electrophoretic separations have been demonstrated on larger devices with 96 sample wells and 48 parallel analysis lanes for high-throughput sizing of double-stranded DNA fragments.[6]

Alternative methods of DNA analysis on microchips include the use of TaqMan real-time PCR in silicon-based reactors for amplification of a variety of DNA and RNA targets,[7−9] detection of pathogenic viruses and bacteria, plant genes, human genetic diseases, single nucleotide polymorphisms,[10,11] and the use of microarrays of oligonucleotide probes for DNA hybridization analysis[12] or minisequencing.[13] While these alternative methods are useful for various high-throughput applications, they do not provide the DNA sizing information required for a number of biological, biomedical, and forensic analyses.

Rapid thermal cycling near 30 s/cycle[6,7,14] and even 17 s/cycle[9] has been demonstrated in silicon microstructures. The short cycle times were achieved by having a small thermal mass and decreasing PCR dwell times. Dwell times down to 1−5 s were used for the melting and annealing steps, and temperature ramp rates of ∼4 °C/s were achieved for cooling[14] and of ∼6 °C/s[9] or even of 30 °C/s[7] for heating. Such a device was used to detect

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bacterial DNA by a real-time TaqMan PCR assay in 7 min.[9] A novel noncontact infrared-mediated thermal cycling in glass chambers enabled a temperature ramping rate of 10 °C/s (heating) and 20 °C/s (cooling) and resulted in cycle times of 17 s/cycle.[15] Continuous flow PCR was performed in a glass chip with a single microchannel that meandered the sample across three temperature zones to fulfill 20 amplification cycles in times ranging from 1.5 to 18.5 min.[16] Fluid manipulations in the latter device were controlled hydrostatically, and the PCR products were collected for conventional analysis by slab gel electrophoresis, which substantially increased the total analysis time.

Although silicon has the advantages of micromachining ease and good thermal conductivity, its optical and electrical properties restrict its use as a substrate for electrophoretic devices. A thin insulative oxide film can be thermally grown on the silicon surface, but these passivation layers do not meet electrical requirements of CE. Nonconductive plastic substrates, such as poly(dimethylsiloxane) and poly(methyl methacrylate), have also been used for fabricating microchips for DNA separations.[17−23] However, their lower thermal conductivity makes them less efficient for thermal cycling and Joule heat removal during electrophoresis. Silica-based substrates (glass and fused silica) are electrically insulating and thermally conductive. Electrokinetic manipulations of reagents and the integration of analysis elements such as miniature reactors, electrophoresis, and optical detection in a single device are feasible.

In this paper, we report an integrated microchip system coupled with a compact thermal cycling assembly based on dual Peltier elements. This system provides rapid and efficient amplification of DNA on-chip followed by electrophoretic analysis of the products on the same chip. The assembly is simple and versatile, enabling easy accommodation of different microchip designs. On-chip concentration of DNA fragments[4] was incorporated into the system, enabling detection of PCR products after performing as few as 10 thermal cycles with a total analysis time of under 20 min. The starting DNA template concentration was 0.3 pM, corresponding to ∼15 molecules per injection volume.

EXPERIMENTAL SECTION

Microfabrication. Microchips were fabricated in white crown glass substrates (SL-4009-2C-AR3-AZ1350, SL-4006-2C-AR3-AZ1350, SL-3W40-225-UP, Hoya Corp., Shelton, CT) by means of standard photolithographic and wet chemical etching techniques, as described previously.[24,27] Bonding of a cover plate to the glass substrate was accomplished using potassium silicate.[4,25] This procedure employs a spin-on layer of diluted silicate solution (KASIL 2130, The PQ Corp., Valley Forge, PA) which serves as an adhesive and forms a thin porous interface between the substrate and cover plate after thermal processing under ambient atmospheric conditions (1 h at 90 °C, temperature ramping to 200 °C at a 0.3 °C/min rate, and annealing at 200 °C for 12 h). The microchannel design is shown in Figure 1. The channels were typically 7−10 μm deep and 40−45 μm wide at half-depth.[24] The separation length was 2.5−3.0 cm. To form reservoirs connected to the microchannels, 3.7-mm diameter holes were drilled (Sonic-Mill, Albuquerque, NM) in the top glass plate (either substrate or cover plate). The typical volume of the reservoirs was 10−20 μL depending on the thickness of the glass plate used.

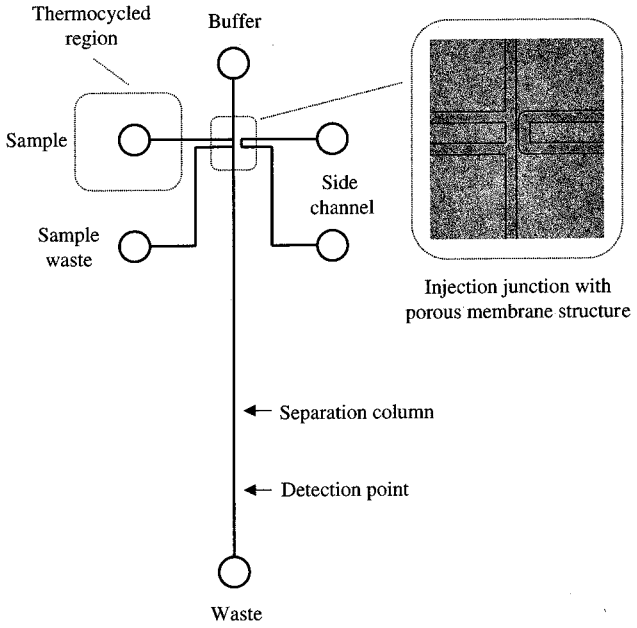

Figure 1. Microchannel design used for integrated rapid PCR analysis on-chip. A porous membrane structure is incorporated in the injection valve (CCD image in the inset) to utilize the on-chip DNA concentration feature.

Microchip Electrophoresis. The electroosmotic flow in the channels was minimized using Bio-Rad Run Buffer (CE-SDS Protein Kit, Run Buffer, Cat. no. 148-5032, Bio-Rad Laboratories, Hercules, CA) as a dynamic coating.[26] After the channels were flushed sequentially with 0.5 M NaOH, deionized H_2O, Bio-Rad Run Buffer, and a final H_2O wash, they were filled with a sieving buffer solution for microchip gel electrophoresis. The coating was stable for multiple injection/separation cycles and provided reproducible separation performance. The simplicity and reliability of this coating procedure allow easy regeneration of the microchannel surface prior to each filling with sieving media and increase chip lifetime by eliminating degradation problems associated with covalent coatings. For DNA fragment sizing, 3% or

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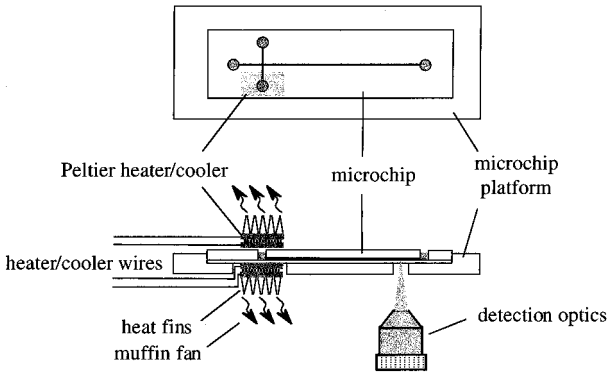

Figure 2. Schematic of the dual Peltier assembly for rapid thermal cycling followed by electrophoretic analysis on-chip.

4% linear polydimethylacrylamide (PDMA) solutions in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA at pH 8.3) were used. PDMA sieving solutions were prepared by polymerization of dimethylacrylamide (Polysciences, Inc., Warrington, PA) in TBE buffer solutions using the ammonium persulfate/TEMED redox system for initiation.[2,3] An intercalating dye, YOPRO-1 (Molecular Probes, Eugene, OR), was added to the polymer solutions at 0.5 μM for the PCR on-chip experiments. A PCR marker (Promega Corp., Madison, WI) containing six DNA fragments of 50, 150, 300, 500, 750, and 1000 base pairs (bp), present at approximately equal weight/volume concentration, was used for PCR verification, product size determination, and estimations of the amplification efficiency. For the laser-induced fluorescence detection, an argon ion laser (488 nm, 6 mW; 543-AP, Omnichrome, Chino, CA) was focused to a spot in the separation channel and the fluorescence signal was collected and processed as previously described.[24,27] Platinum electrodes provided electrical connections from the power supply to the reservoirs. The data acquisition and voltage switching operations were computer-controlled using in-house programs written in LabView (National Instruments, Austin, TX).

For the conventional injection/separation cycle, without on-chip concentration (described below), the sample was first loaded into the double-tee injection region[24,28] by applying a high voltage at the sample waste and grounding the sample reservoir for 100 s. The buffer and waste reservoirs had no potentials applied since the low diffusion coefficients of DNA fragments in the polymer sieving medium resulted in no substantial increase in the injection plug length or deterioration of the separation performance. To inject the sample plug into the separation channel, the relative potentials were switched to 0, 1, 0.3, and 0.5 at the buffer, waste, sample, and sample waste reservoirs, respectively.

Rapid Thermal Cycling On-Chip. Figure 2 depicts a schematic of the compact system that integrates rapid PCR amplification and subsequent product analysis on a single chip. The system uses dual Peltier thermoelectric elements (model DT-4, Marlow Industries, Inc., Dallas, TX) to sandwich the chip in the cycled regions. A drop of mineral oil on the bottom Peltier element ensured good thermal contact with the cycled region of the chip (see Figure 1). The top thermoelectric element was not placed in direct contact with the chip in order to eliminate the requirement of sealing the top reaction reservoir against contact-driven capillary wicking. Thus, the top assembly was positioned over the chip using 0.5-mm-thick plastic strips as spacers and therefore providing only radiative heat transfer to the cycled region of the chip. Thermoelectric elements were driven using a DC power supply (model E3614A, Hewlett Packard, Santa Clara, CA) in voltage-following mode, providing full power (0−6 A at 0−8 V) using an analog output control signal from the computer. Solid-state switching provided current reversal through the Peltier devices for active heating and cooling operation. Twelve-volt microprocessor-chip cooling fans and integral heat sinks provided heat removal from the Peltier elements. Thermocouple elements (Type K chromel/alumel wire, 36 gauge, Hoskins Mfg. Co., Hamburg, MI) were embedded into the surface of each Peltier element for temperature monitoring and control. The surface temperature of the bottom unit was used as the input for a proportional/integral/derivative (PID) control algorithm to control the power to both Peltier units.

The bottom unit thus closely followed the programmable set points used during thermocycling ($< \pm 0.5$ °C). Chip cycling performance was predominantly dependent on the bottom Peltier unit, due to its direct thermal contact with the bottom of the chip. A 0.4-mm-thick HOYA glass cover plate, bonded to the bottom surface of the chip substrate, provided the bottom surface of the reaction wells. The top Peltier unit, however, was allowed to significantly overshoot and undershoot set-point values, as the radiative heat transfer mode from this element was not coupled strongly to chip cycling performance. This conductive/radiative control scheme was slower than conductive/conductive sandwiching of the chip between the Peltier elements with intimate contact with both sides of the chip or with methods where a control thermocouple is directly immersed into the PCR reaction mix. However, this control scheme provided the most flexible and reliable thermal cycling performance, resulting in fast, critically damped thermal cycling and eliminating the potential for boiling off fluid which can occur with very minor overshooting at PCR denaturing temperatures. It also reduced the potential for PCR reaction mix contamination which can occur using immersed control elements. This conductive/radiative approach also simplified the thermal cycling procedure since it eliminated the reservoir sealing step and consequent problems related to reaction inhibition by sealing adhesives and thermally induced fluid pumping.

In practice, thermal ramp rates at the control points were typically 20−30 °C/s, with the reaction mix ramping at 2 °C/s (heating) and 3−4 °C/s (cooling). The ramp rates can be estimated from the data in Figure 3 presenting probe temperature as a function of time. The reaction mix temperature was monitored by immersing a thermocouple into the solution. Thus, ramp intervals between set points were typically 1−2 s, with hold periods of only 10−30 s, providing complete volume equilibration at specific PCR set points. Overshooting at the bottom thermoelectric element (thin solid line in Figure 3), which is more pronounced at the annealing step, was designed to speed up temperature ramping in the reaction solution (dashed line in Figure 3). The heating and cooling capacities of the elements were sufficient for control over the full temperature range of PCR, from 30 to 96 °C during cycling and down to 4 °C for long-term storage following cycling.

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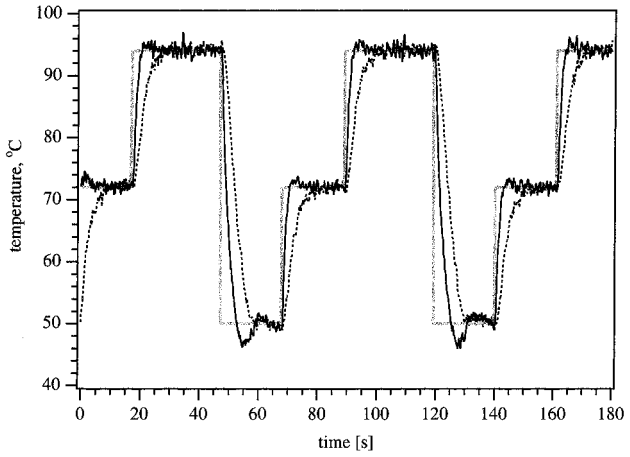

Figure 3. Thermoelectric assembly performance. Temperature versus time for the PCR amplification (199-bp fragment of λ phage DNA): set-point temperature (gray thick solid line), bottom Peltier element control probe (black thin solid line), reaction mix probe (dashed line).

PCR Amplification. The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 250 μg/mL bovine serum albumin (BSA), 200 μM each deoxynucleotide triphosphate, 0.1−1.0 μM each primer, 25 units/mL AmpliTaq DNA polymerase (Perkin-Elmer), and 10 ng/mL λ phage DNA, which corresponded to a starting template copy number of less than 15 per 80-pL injection volume. YOPRO-1 was added to the PCR cocktail at a concentration of 0.5 μM. Preliminary experiments showed that the presence of this intercalating dye does not inhibit the amplification efficiency. A 199-bp segment of λ phage DNA was chosen for amplification, and the primer set used was 5′-GGT TAT CGA AAT CAG CCA CAG CGC C-3′ and 5′-GGA TAC GTC TGA ACT GGT CAC-3′ (Gibco Life Technologies, Inc., Grand Island, NY). All microchannels and reservoirs, excluding the reaction well (sample well in Figure 1), were filled with PDMA sieving solution. The reaction reservoir was first treated with bovine serum albumin (BSA) solution (2.5 mg/mL) for 2−3 min to passivate the inner glass surface of the milled reservoir. The BSA was then removed from the well and replaced with 6 μL of PCR reaction mixture. To prevent evaporation all wells were topped with 10−12 μL of Chill-out 14 Liquid Wax (MJ Research, Inc.). For amplification, the temperature steps were 94, 50 and 72 °C with hold times of 30, 20 and 25 s, respectively, resulting in ∼ 1.25 min/cycle. No special attempts to minimize the PCR dwell times were made at this point, but preliminary experiments indicate the possibility of speeding up the process by several fold depending on the sample volume, reservoir geometry, glass cover plate thickness, thermal conductivity of a substrate material, and specific characteristics of a given PCR system.

Upon completion of the PCR program, the upper heater/cooler assembly was removed, electrodes inserted into the reservoirs, and electrokinetic manipulations performed to analyze the products. For comparison of cycling efficiency, amplification experiments were also performed by thermal cycling of the entire chip in a commercial thermocycler, as described previously.[2]

On-Chip Concentration of DNA Fragments. To incorporate on-chip DNA concentration[4] into this integrated system, the microchip design shown in Figure 1 was utilized. The main and side channels were separated by a distance of 3−5 μm. Electrical contact between the two separated channels is established through the porous, semipermeable polysilicate membrane formed during the silicate bonding process.[4,25] This allows conduction of ionic current but prevents large DNA molecules from crossing the membrane. As a result, a substantial concentration of DNA takes place in the main channel adjacent to the membrane. The concentrated plug can then be injected into the separation channel and electrophoretically analyzed. In this study, PCR products were typically concentrated by applying 1 kV between the sample and side channel reservoirs for 1−2 min with no potentials applied to the other reservoirs. To inject the concentrated plug, the voltages were reconfigured for the separation mode with the relative potentials at the buffer, waste, and sample reservoirs being 0, 1, and 0.3, respectively, and the other reservoirs floating. The microchip design used enabled sample analysis by either conventional injection without preconcentration, as described above, or concentration of DNA fragments at the porous membrane junction prior to injection. This feature allowed a direct evaluation of the concentration enhancement obtained employing the porous membrane structure in the injection valve.

RESULTS AND DISCUSSION

The integrated microchip-based thermal cycling system demonstrates the feasibility of rapidly performing integrated PCR and CE analyses in microchips. The temperature cycling performance of the dual Peltier assembly is reflected in Figure 3, which shows temperature profiles for three cycles of the program used. Although the temperature at the bottom Peltier element control probe (black thin solid line in Figure 3) indicates ±2 °C fluctuations around the set points, the reaction mix itself (dashed line in Figure 3) tends to reduce the temperature variations and follows the model profile (gray thick solid line) for amplification of the 199-bp segment of λ DNA with good consistency from cycle to cycle, maintaining an accuracy of ±0.5 °C at each set point. Active heating and cooling of the system provides very fast ramp rates between the set points, typically 20−30 °C/s. However, the complete volume equilibration of the reaction mixture requires hold periods of 10−30 s at the specific PCR steps due to slower ramping in the solution (dashed line in Figure 3).

Successful DNA amplification is dependent upon the preliminary treatment of PCR reservoirs as well as the reaction mixture volume. A BSA solution (2.5 mg/mL) was placed in the reaction well for 2−3 min prior to the PCR cocktail to coat the glass surface and prevent adsorption of PCR polymerase or nucleic acids. Optimum thermal uniformity during heating/cooling cycles resulting in a good PCR efficiency was achieved using an ∼5−7 μL volume of the PCR cocktail in 3.7-mm diameter reaction wells.

Figure 4a shows the electropherogram of the 199-bp PCR product, amplified and electrophoretically analyzed on the same chip without preconcentration. Total analysis time was approximately 30 min and included 25 rapid thermal cycles and electrokinetic injection/separation manipulations. For determining the length of unknown amplified products or verifying the sizes of known target sequences, PCR products were mixed and coelectrophoresed with size markers as demonstrated in Figure 4b for the 199-bp product. The latter profile was obtained by simply adding a PCR marker solution to the sample reservoir containing the PCR product. However, electrokinetic manipulations on-chip

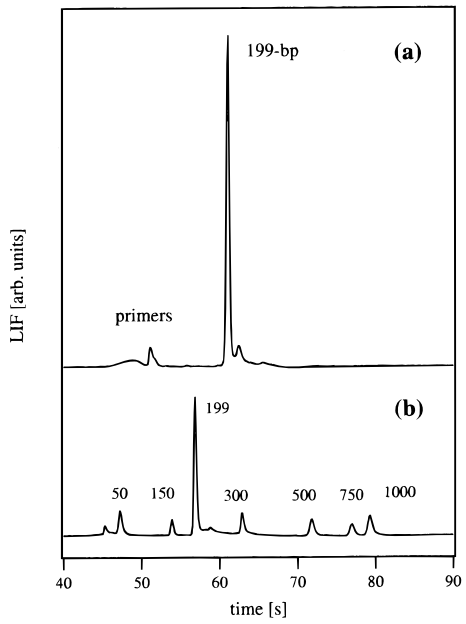

Figure 4. Electrophoretic analysis following on-chip rapid amplification of a 199-bp fragment of λ phage DNA after (a) 25 thermal cycles and (b) after being mixed with PCR marker. Electrophoretic conditions: 4% PDMA in 0.5× TBE sieving medium, 2-cm separation distance, 200 V/cm field strength.

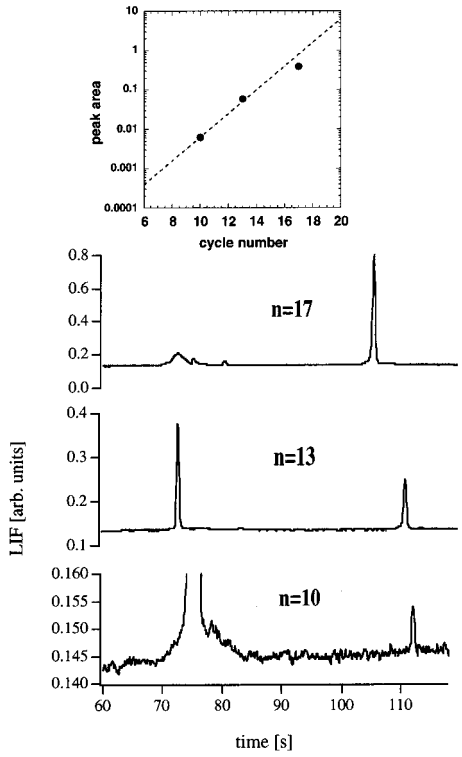

Figure 5. Electrophoretic analysis following on-chip rapid amplification of a 199-bp fragment of λ phage DNA at different numbers of thermal cycles (n). Electrophoretic conditions: 4% PDMA in 0.5× TBE sieving medium, 3-cm separation distance, 190 V/cm field strength. Inset: peak area as a function of cycle number. Dashed line represents an ideal exponential PCR amplification given by $y = 6.0 \times 10^{-6}2^n$.

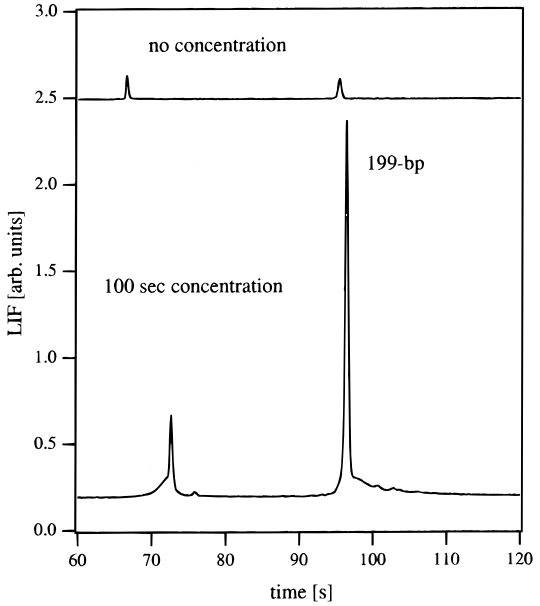

Figure 6. Electrophoretic analysis with and without concentration of PCR product after 20 cycles. Electrophoretic conditions: same as in Figure 5.

allow control of multiple sample wells for mixing and co-injecting PCR products and markers in the desired proportion by programming the applied potentials. We have previously shown[3] that PCR products from multiple sample wells can be analyzed in a single electrophoresis channel either individually or simultaneously by on-chip mixing. Moreover, sizing markers can be mixed on-chip with samples for accurate fragment sizing.

The rapid on-chip PCR amplification efficiency attained was comparable to that obtained in standard tubes using a commercial thermocycler. The typical product yield for a chip was 70−100% of tube PCR, based on both slab gel and microchip electrophoresis analysis. The average efficiency per cycle of on-chip PCR amplification was estimated to be ~58%. This estimation was made on the basis of the initial concentration of a 199-bp segment in the template (~40 pg/mL for 10 ng/mL of λ phage DNA) and comparing peak areas for the PCR product and marker peaks of known concentration. An average increase of 10^5 over the initial target region concentration was achieved in 25 cycles, which corresponds to ~1.58-fold amplification per cycle.

Figure 5 depicts electrophoretic profiles of the PCR product obtained after 10, 13, and 17 cycles on-chip. The relatively high efficiency of the thermal cycling method developed for on-chip PCR combined with high detection sensitivity allowed detection of the product after as few as 13 cycles. In some cases, a small peak was observed after only 10 cycles. However, the efficiency of on-chip DNA amplification can vary significantly depending on the condition of the particular reservoir's inner glass surface, as well as other experimental parameters. The earlier peaks appearing in the electropherograms in Figure 5 are due to the primers which are present at relatively high levels at low cycle numbers and decrease with each cycle (cyanine intercalating dyes can bind to both double- and single-stranded DNA with similar affinity[29]). The inset in Figure 5 shows the PCR product peak area versus cycle number derived from the three electropherograms. The

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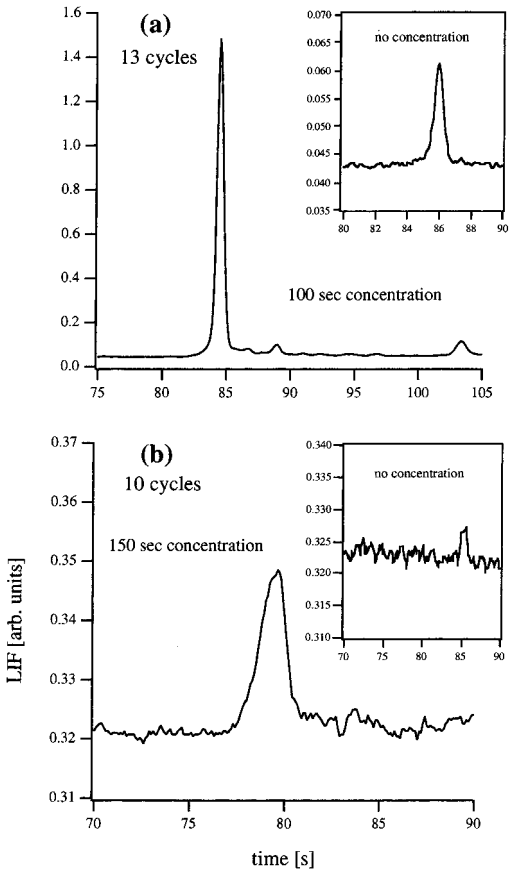

Figure 7. Electrophoretic analysis of PCR products after (a) 13 and (b) 10 cycles. Electrophoretic conditions: 4% PDMA in 0.5× TBE sieving medium, 3-cm separation distance, 215 V/cm field strength.

dashed line is the ideal PCR amplification fitting the equation, $y = k2^n$, where n is the number of cycles, and the proportionality constant, k, was determined from the $n = 10$ peak area. The proximity of the experimental data to the theoretical curve reflects efficient PCR amplification in the integrated microchip format at low cycle numbers, where primer concentration is not limiting.

To further enhance the performance of the integrated microchip analysis, a microfabricated injection valve with a porous membrane structure was incorporated to concentrate DNA prior to electrophoretic separation and detection. Initially, PCR product analysis at a low number of thermal cycles, using the combination of on-chip DNA amplification and concentration techniques, was demonstrated by thermally cycling the entire chip in a commercial instrument as described previously.[2] Following 12 cycles, the generated product could be detected only after applying on-chip preconcentration, and the signal increased with longer concentration times. An example of an integrated analysis is shown in Figure 6. On-chip generated PCR product was run with and without concentration for 100 s prior to the injection after 20 rapid thermal cycles. There is also an indication that the concentration membrane discriminates against the primers relative to the larger PCR products. Signal enhancement of approximately 25-fold was achieved for the 199-bp PCR product versus a factor of 5 for the primer peak. Figure 7a demonstrates a 70-fold concentration for a 199-bp product after 13 cycles. Implementing the on-chip concentration step enabled detection of the PCR product after just 10 cycles and shortened the total analysis time to under 20 min (Figure 7b). Lack of consistency of the on-chip concentration performance leads to a variability in the degree of enhancement of DNA analysis achieved in the experiments. Work on the refinement of the microfabricated porous structures and optimization of this system is under way. The porous membrane structures may also provide some size fractionation of reaction mixtures, as indicated above, and a means for desalting samples prior to electrophoretic analysis.

In conclusion, an integrated system for rapid PCR-based DNA analysis was developed to couple a compact thermal cycling assembly based on dual Peltier elements to microchip capillary electrophoresis analysis. The system is easily adaptable to various chip designs and demonstrates the feasibility of portable microchip-based instrumentation platforms. The incorporation of a microfabricated porous membrane structure provided additional sample amplification, allowing the use of reduced numbers of PCR cycles, enabling complete analysis in under 20 min. More sophisticated detection strategies, such as those recently demonstrated coupling single-chromophore[30] and single-molecule[31] detection to microchip-based electrophoretic separations, could further reduce the required number of PCR cycles needed for genetic analysis to yield yet higher information generation rates.

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