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Functional Integration of PCR Amplification and Capillary Electrophoresis in a Microfabricated DNA Analysis Device

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Microfabricated silicon PCR reactors and glass capillary electrophoresis (CE) chips have been successfully coupled to form an integrated DNA analysis system. This construct combines the rapid thermal cycling capabilities of microfabricated PCR devices (10 °C/s heating, 2.5 °C/s cooling) with the high-speed (<120 s) DNA separations provided by microfabricated CE chips. The PCR chamber and the CE chip were directly linked through a photolithographically fabricated channel filled with hydroxyethylcellulose sieving matrix. Electrophoretic injection directly from the PCR chamber through the cross injection channel was used as an "electrophoretic valve" to couple the PCR and CE devices on-chip. To demonstrate the functionality of this system, a 15 min PCR amplification of a *β*-globin target cloned in M13 was immediately followed by high-speed CE chip separation in under 120 s, providing a rapid PCR−CE analysis in under 20 min. A rapid assay for genomic *Salmonella* DNA was performed in under 45 min, demonstrating that challenging amplifications of diagnostically interesting targets can also be performed. Real-time monitoring of PCR target amplification in these integrated PCR−CE devices is also feasible. Amplification of the *β*-globin target as a function of cycle number was directly monitored for two different reactions starting with 4×10^7 and 4×10^5 copies of DNA template. This work establishes the feasibility of performing high-speed DNA analyses in microfabricated integrated fluidic systems.

The development of enhanced DNA amplification and analysis devices and methods having improved speed and reduced reagent volumes will be critical for the completion of the Human Genome Project and for the subsequent utilization of this sequence information. The polymerase chain reaction (PCR)[1] has advanced DNA amplification dramatically but currently requires long cycling times because of the large thermal mass of typical systems with concomitant slow heating and cooling rates. These problems have been addressed in part by placing samples in small glass capillaries and using heated air to drive the cycling,[2] but the introduction and removal of small sample volumes and the handling of capillaries can be problematic. Similarly, while the throughput of conventional slab gel electrophoresis can be increased by using thinner slab gels[3] or multiplex labeling,[4,5] the labor-intensive steps of gel preparation, as well as sample loading, are still necessary. We[6−12] and subsequently others[13,14] have shown that capillary array electrophoresis can be used to dramatically increase the throughput of DNA sequencing[6−8,11−13] and fragment sizing[9,10,14] separa-

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tions, compared to conventional slab gel electrophoresis. However, the preparation and manipulation of large numbers of capillaries can be difficult, and sample introduction can be inefficient. It is evident that the most challenging and important issue in the submicroliter miniaturization of DNA analyses is the integration of the various amplification and analysis steps.

Although a variety of microfabricated structures for PCR[15−19] and electrophoresis[19−23] have been proposed and in some cases demonstrated, the integration of these two important processes into one functional system has not occurred. PCR amplification has been performed inside 4−12 μL microfabricated Si-glass chambers placed in a larger thermal cycler,[16,18] on a 25 μL drop of solution on top of a microfabricated heater,[19] and inside 20−50 μL microfabricated Si chambers with integrated heaters,[15,17] but in each case the sample was removed for conventional external analysis. The Si chambers with integrated heaters[15,17] are advantageous because they match the size of the thermal cycler to the sample volume, thereby reducing the time for amplification to as low as 30 s/cycle. Microfabricated capillary electrophoresis (CE) chips have been fabricated on glass substrates and used to perform separations of fluorescent dyes,[20,22] labeled amino acids,[24,25] and short oligonucleotides.[26] In our own work, we demonstrated that CE chips can be used to perform very rapid (<120 s) separations of DNA restriction fragment digests and PCR products.[23] We also demonstrated for the first time that DNA sequencing fragments could be separated on CE chips with single-base resolution.[27] The integration of the amplification and analysis of DNA on-chip will require the development of methods for reliably fabricating multiple components on a single microdevice and controlling the transfer of DNA between systems. For example, electroosmotic flow,[28,29] electrophoresis,[30] and thermocapillary pumping[19] have been used to move and mix solutions on chips. Although many of these individual components have been developed, it has not been demonstrated that two fundamentally different and important device functions such as PCR and CE can be functionally integrated onto a single device.

We demonstrate here apparatus and methods that permit the direct integration of microfabricated PCR and CE components on a single microdevice. The functionality of this construct is demonstrated through PCR−CE analysis of β-globin in under 20 min and PCR−CE analysis of *Salmonella* DNA in under 45 min. Injection via an "electrophoretic valve" is performed, where the viscous hydroxyethylcellulose (HEC) sieving matrix in the interconnecting channels on the CE chip prevents the flow of PCR reagents into the separation channel during thermal cycling, while still allowing us to electrophoretically drive DNA out of the PCR chamber for CE analysis. Finally, we show that these integrated devices offer the unique capability to perform real-time monitoring of PCR amplification. This work demonstrates the feasibility of fabrication and use of complex, integrated DNA analysis microdevices.

EXPERIMENTAL SECTION

Microfabrication. Photolithography and etching of the CE chips was performed at the University of California, Berkeley Microfabrication Laboratory as described previously.[23] Briefly, two CE systems consisting of an injection channel intersecting with a separation channel were fabricated on each device (Figure 1A). The injection channel connected reservoirs 1 and 3 and the separation channel connected reservoirs 2 and 4. The separation channels were 100 μm wide, 8 μm deep, and 46 mm long, with a distance of 40 mm from the injection region to the end of the channel. The injection channels were 50 μm wide, 8 μm deep, and 12 mm long, with a distance of 2 mm from reservoir 3 to the intersection with the separation channel. After alignment, the etched bottom plate was thermally bonded to the top glass substrate which had access holes drilled over the etched reservoirs. The drilled holes for reservoir 3 were 1.5 mm in diameter, while all other holes were 0.8 mm in diameter.

The PCR chambers were fabricated at the Lawrence Livermore National Laboratory (LLNL) Microtechnology Center from cleaned 1.0 mm thick double-sided polished silicon 100 wafers.[17] After low-pressure chemical vapor deposition (LPCVD) of silicon nitride (1 μm), the nitride was photolithographically patterned and reactive ion etched down to the silicon. The photoresist was removed, the silicon was etched to a depth of 850 μm with KOH to form the chambers, and the wafers were cleaned again. LPCVD of polysilicon (3000 Å) was boron doped to a sheet resistance of 400 Ω/square. The polysilicon was photolithographically patterned and etched, and the photoresist was removed. The polysilicon was again photolithographically patterned for the heater contacts followed by E-beam deposition of 2500 Å of gold (5 Å/s) on top of 100 Å of titanium (2 Å/s); after lift-off, the wafer was sawed. The chambers were formed by bonding two of the identical pieces together with polyimide (Epo-Tek 600, Epoxy Technology) and curing completely. The etched regions formed a tube of hexagonal cross section, extending the length of the reactor. A 30 AWG Teflon insulated thermocouple (type K, Omega, Stamford, CT) was affixed to one side of the reactor with

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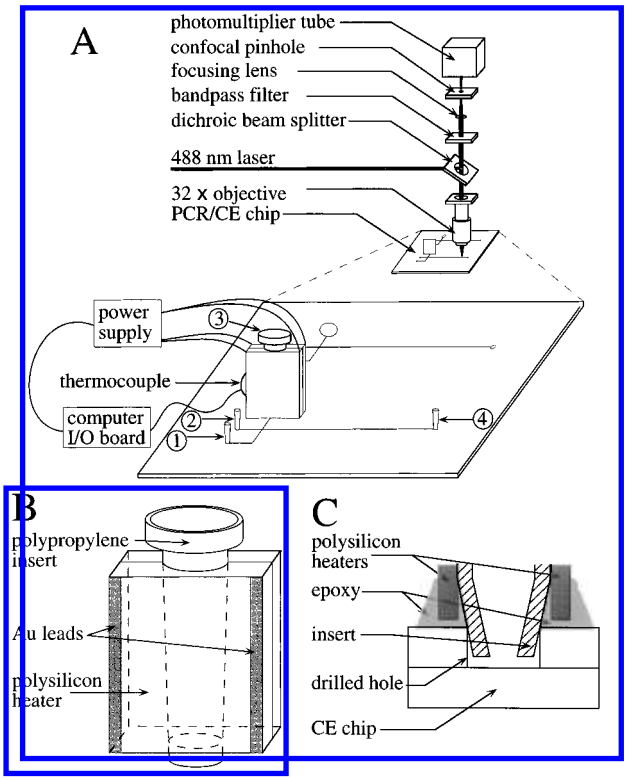

Figure 1. Schematic of the integrated PCR−CE microdevice. (A) Laser-excited confocal fluorescence detection apparatus and an integrated PCR−CE microdevice. (B) Expanded view of the microfabricated PCR chamber. (C) Expanded cross-sectional view of the junction between the PCR and CE devices. The size of the epoxy-filled gaps is exaggerated for viewing clarity.

thermally conductive epoxy (Tra-Bond BB2151, Tra-Con, Medford MA). Small wires were attached to the Au heater leads using conductive epoxy (Planned Products, Santa Cruz, CA). Disposable, thin-walled polypropylene liners for the PCR devices, fabricated at LLNL, were inserted down the length of the hexagonal-shaped tube in the reactor. The inserts were 2 cm tall narrow tubes with 1.0 mm inside diameter and 1.7 mm outside diameter, tapered to 1.5 mm outside diameter at the bottom, and expanding to a wider 4.0 mm opening at the top. The volume of solution held by an insert inside the PCR chamber was ~20 μL. These plastic inserts helped avoid contamination of subsequent PCR experiments, as well as failure of PCR due to adsorption of reagents to the Si surface of the reactor.

Coupling the PCR and CE Chips. The plastic liner was inserted in the PCR device so that it extended ~1 mm out through the bottom hole in the Si chamber. The protruding end was inserted into the drilled hole corresponding to reservoir 3 in the CE chip so there was a snug fit. Fast setting epoxy (Double Bubble, Hardman, Belleview, NJ) was applied around the connecting region to form a leakproof union of the devices (Figure 1C). Successful, leakproof integration of the devices occurred in 19 of the 25 bondings. The PCR−CE microdevices were disassembled and then reassembled with a clean insert between subsequent amplifications to avoid contamination.

PCR Amplification and Electrophoresis. PCR was performed on a 268 bp β-globin target cloned in M13 using reagents provided by Roche Molecular Systems (Alameda, CA). Standard 1× PCR buffer (50 mM KCl and 10 mM Tris-Cl, pH 8.3) with 3 mM $MgCl_2$; 10% glycerol; 400 μM dATP, dGTP, dCTP, and dTTP; 0.5 μM GH20 primer and PC04 primer; 2.5 units of *Taq* polymerase; and 10^6 or 10^8 starting copies of β-globin DNA template in 50 μL of solution were used. The rapid PCR experiments on β-globin were performed with 30 cycles at 96 °C for 2 s, 55 °C for 5 s, and 72 °C for 2 s. Two-step thermal cycling of β-globin was carried out with steps of 96 °C for 30 s and 60 °C for 30 s. Genomic *Salmonella* DNA for PCR amplification was obtained from Dr. William Laegreid of the USDA/ARS. Amplification was performed using the S18 and S19 primers with 500 ng of genomic *Salmonella* DNA per 50 μL of solution as described previously.[31] Thermal cycling of genomic *Salmonella* DNA was performed with 35 cycles at 95 °C for 10 s, 56 °C for 15 s, and 72 °C for 20 s. Control amplifications were carried out in a Perkin-Elmer 480 thermal cycler (Foster City, CA).

The CE separation medium was 0.75% (w/v) HEC in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) with 1 μM of the intercalating dye thiazole orange to fluorescently label the DNA on-column.[23] The PCR−CE microdevices were filled with HEC buffer via reservoir 4 (Figure 1A) by forcing the solution through all the channels of the assembled PCR−CE microdevice using a syringe. The PCR chamber formed by the insert and microfabricated heater coupled to the CE chip was rinsed with water five times, primarily to remove any EDTA introduced into the chamber when the channels were filled with HEC buffer. The PCR solution (20 μL) was introduced into the rinsed chamber, which was then loosely capped to reduce evaporation during thermal cycling. The other three buffer reservoirs were formed by inserting cutoff pipet tips in the drilled holes and filled with HEC solution. Electrical contacts were established on the CE chip by inserting Pt wires into the reservoirs; however, the wire in the PCR chamber (reservoir 3) was removed during thermal cycling to avoid inhibition of the PCR amplification. No sample handling was necessary for the subsequent analysis. After PCR, 300 V/cm was applied between reservoirs 1 and 3 for 10 s with β-globin (20 s for *Salmonella*) to inject the amplified DNA into the separation channel; separation was performed by applying 200 V/cm between reservoirs 2 and 4.[23] Two standard DNA sizing ladders, ϕX174 *Hae*III (New England Biolabs, Beverly, MA) and a 50 bp ladder (Pharmacia, Piscataway, NJ), were used to verify sizes of the PCR products.

Instrumentation. Laser-excited confocal fluorescence detection was performed as described previously[23,27] with minor modifications. Briefly, the 488 nm line from an argon ion laser was focused within the channel using a 32× NA 0.4 long working distance objective (LD Achroplan 440850, Carl Zeiss, Thornwood, NY). Fluorescence was collected by the objective and passed through a dichroic beam splitter and a band-pass filter, followed by spatial filtering with a confocal pinhole prior to photomultiplier detection. Amplified photoelectron pulses were converted to an analog signal (0−0.3 V) using a home-built integrator. The analog signal was sampled at 10 Hz with a 16 bit ADC board (NB-MIO-16XL-18, National Instruments, Austin, TX) controlled by a program written in LabVIEW running on a Macintosh Quadra 700 computer.

The PCR chips were thermally cycled with an electronic controller based on a pulsed width modulator (PWM), designed and fabricated at LLNL. The PWM was controlled using a program written in IgorPro (Lake Oswego, OR) via an ADC board (National Instruments) in a Macintosh Quadra 650 computer.

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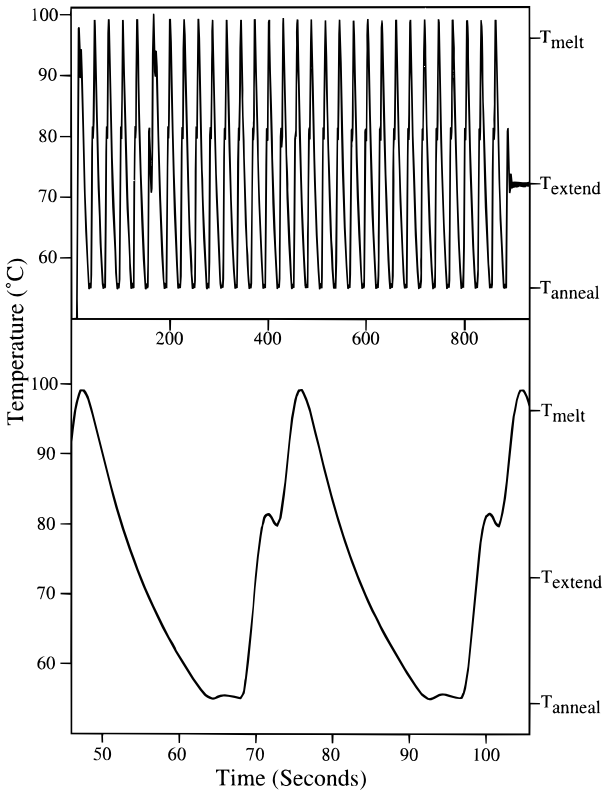

Figure 2. (top) Temperature of the PCR device as a function of time during a 15 min amplification of β-globin. The temperature profile was 96 °C for 2 s, 55 °C for 5 s, and 72 °C for 2 s, with a final 72 °C extension step of 30 s. (bottom) Expanded view of the second and third cycles in the experiment. Typical cooling rates from melting to annealing temperature are 2.5 °C/s; typical heating rates are ~10 °C/s, so that one cycle is completed in less than 30 s.

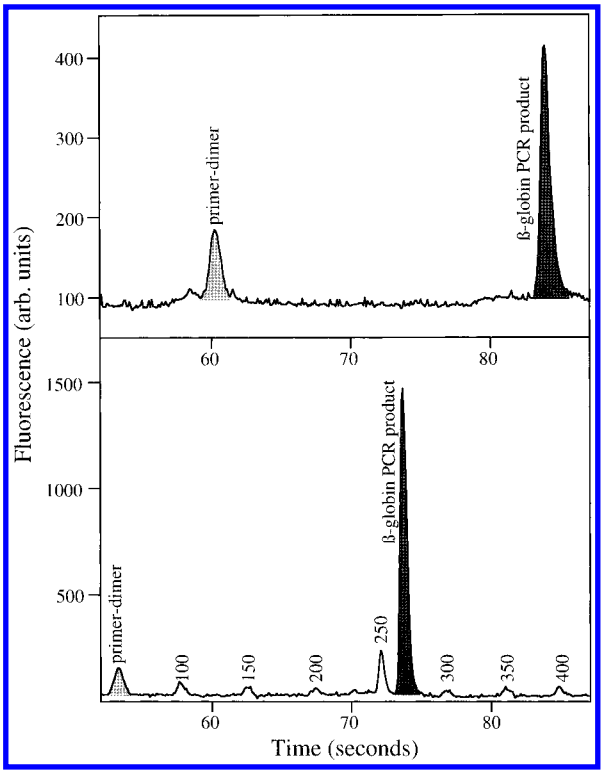

Figure 3. High-speed analysis of β-globin on the integrated PCR−CE microdevice. (top) Chip CE separation of the β-globin PCR product performed immediately after the 15 min thermal cycling shown in Figure 2. The primer−dimer peak (light gray) is visible at 60 s, and the PCR product peak (dark gray) appears at 83 s. Total time for analysis in the integrated PCR−CE microdevice was less than 20 min. (bottom) CE chip sizing of the β-globin PCR product (1:100 dilution) after external mixing off-chip with a 50 bp ladder (1 ng/μL). The primer−dimer peak and the 50 bp peak in the ladder overlap in this separation. The sizing was performed in a different CE chip that did not have a PCR chamber affixed to it.

During heating, the computer turned the PWM "full on" until the temperature of the reaction chamber reached the set point; then the PWM maintained the temperature to an accuracy of ±0.5 °C. When the heating cycle was completed, the PWM turned off, and the reaction chamber cooled passively. The PWM started to control again when the temperature reached the new set point. To speed the cooling step, a fan circuit was enabled and air was drawn along the heater surfaces of the reaction chamber. Prior to use, each PCR chamber was thermally calibrated by filling it with water and measuring the temperature of the reactor's exterior and the temperature of the liquid inside the insert using a calibrated thermocouple for temperature set points from 45 to 95 °C at 10 °C intervals. This allowed the computer program to compensate for any difference between the solution temperature and the measured reactor exterior temperature. Additionally, the thermal cycling soak times were optimized to compensate for the small lag time between solution temperature and reactor exterior temperature, due to the reduced thermal conductivity of the insert.

RESULTS AND DISCUSSION

Figure 2 documents the reactor temperature as a function of time for the rapid PCR−CE analysis of β-globin cloned in M13. The time for performing 30 cycles of amplification is 900 s. The lower panel shows the temperature profile for cycles 2 and 3 in this experiment in greater detail. Cooling from the melting temperature (96 °C) to the annealing temperature (55 °C) takes ~16 s, corresponding to a cooling rate of 2.5 °C/s; the heating rates with the microfabricated PCR device are even faster, ~10 °C/s. The time required for one complete cycle of melting, annealing and extension is between 25 and 30 s, compared to typical heating and cooling rates of ~1 °C/s[1] and 2−6 min cycle times for conventional thermal cyclers.

The top panel of Figure 3 presents the results of a high-speed analysis of the β-globin target on the integrated PCR−CE microdevice. After thermal cycling, the sample was immediately injected electrophoretically; *no pipetting or manual transfer of the mixture was required.* The smaller primer−dimer peak was detected at 60 s, and the PCR product peak was detected ~83 s after injection. *The entire time for integrated PCR−CE analysis of the β-globin target was less than 20 min.* The bottom portion of Figure 3 presents an electropherogram of the same PCR product mixture performed on a separate CE chip following external 1:100 dilution of the product in water and spiking with a 50 bp sizing ladder. The PCR product peak and primer−dimer are detected along with the peaks of the 50 bp ladder. The β-globin PCR product was sized to 266 bp using the known peaks of the standard ladder, confirming that the desired target was amplified (actual size 268 bp). The fragment migration times differ between these two runs because the separations were performed on different CE chips.

Figure 4 demonstrates that the PCR−CE microdevice can also be used for the analysis of genomic *Salmonella* DNA. Immediately following thermal cycling, the sample was electrophoretically injected for 20 s, and the separation shown in the

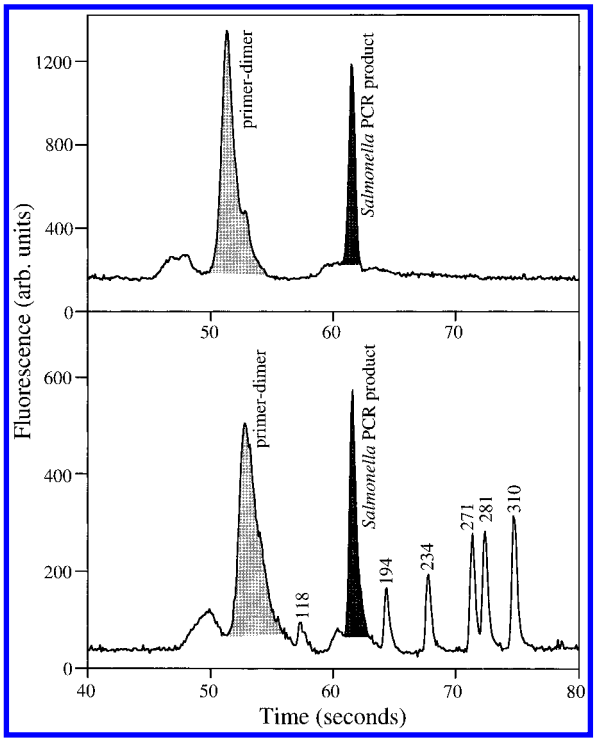

Figure 4. High-speed integrated PCR−CE microdevice assay of genomic *Salmonella* DNA. (top) Chip CE separation of the *Salmonella* PCR product was performed immediately following a 39 min PCR amplification in the integrated PCR−CE microdevice. The primer−dimer peak (light gray) appears at 51 s and the PCR product peak (dark gray) appears at 61 s. Total analysis time for the *Salmonella* sample using the integrated PCR−CE microdevice was under 45 min. (bottom) Sizing of the *Salmonella* PCR product (1:100 dilution) using ϕX174 *Hae*III DNA (1 ng/μL) in a separate CE chip.

top panel was obtained. The PCR product peak is visible at 61 s, along with the primer−dimer peak at 51 s. *This integrated PCR−CE analysis for Salmonella required less than 45 min from initiation of PCR to the completion of the separation.* The lower panel in Figure 4 shows an electropherogram of the PCR product mixture, diluted 1:100 in water and spiked with ϕX174 *Hae*III DNA, which was performed in a separate CE chip. The PCR product peak and the primer−dimer are visible among the peaks of the ϕX174 DNA standard. The excellent resolution and high speed in these CE chip separations are evidenced by the near-baseline resolution of the 271 and 281 bp fragments in under 75 s. The PCR product peak was sized to 164 bp versus the peaks of the standard, which confirms that amplification of the desired target occurred (actual size 159 bp).

One of the unique capabilities of our integrated PCR−CE microdevice is the ability to perform real-time monitoring of PCR amplification. Figure 5 presents sequential electropherograms obtained of the *same* reaction mixture after 15, 20, 25, and 30 cycles of amplification of the β-globin target. After 15 cycles, the PCR product peak is rather small, and no primer−dimer is visible. The target peak continues to grow after 20 and 25 cycles, and the primer−dimer peak also is detected. By 30 cycles of amplification, the product peak growth has plateaued, and in addition to the primer−dimer peak, an artifact peak at 57 s is also detected. Figure 6 plots the logarithm of the PCR product peak area versus cycle number for this experiment, as well as for a similar experiment with 100-fold fewer starting copies of the β-globin template. The signal approaches a plateau region as the reagents are consumed at higher cycle numbers for both 4×10^7

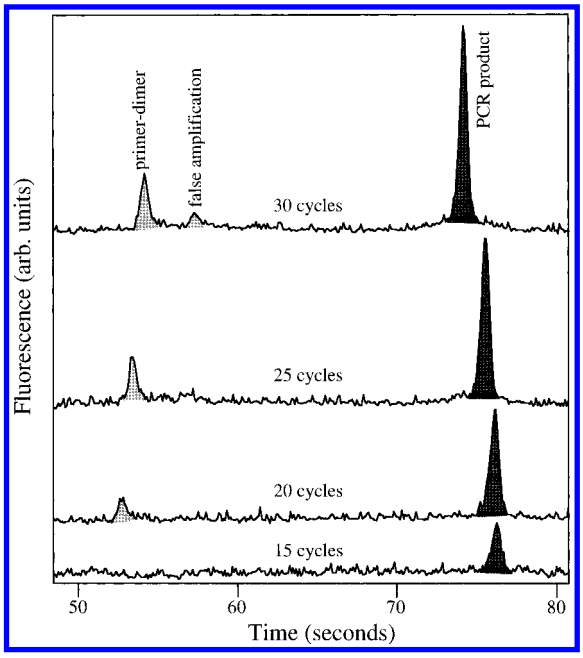

Figure 5. Real-time analysis of a β-globin PCR amplification using an integrated PCR−CE microdevice. Chip CE separations of the same sample were performed sequentially in the integrated PCR−CE microdevice after 15, 20, 25, and 30 cycles at 96 °C for 30 s and 60 °C for 30 s. The PCR product peak is shaded with dark gray and the false amplification and primer−dimer peaks are shaded with light gray.

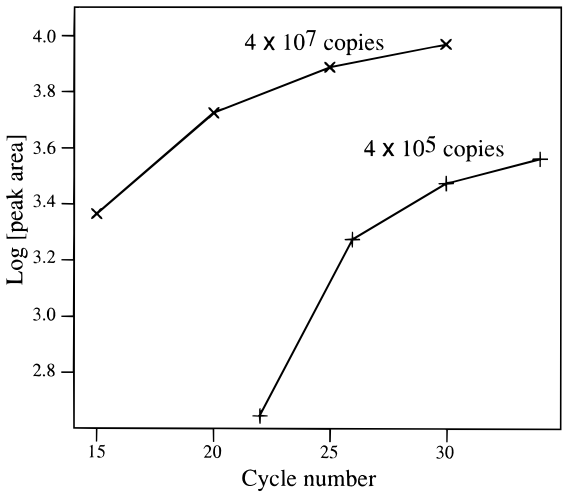

Figure 6. PCR amplification of the β-globin target as a function of cycle number measured in an integrated PCR−CE microdevice. The logarithm of PCR product peak area as a function of cycle number for one sample containing 4×10^7 starting copies ($\times$) and another sample containing 4×10^5 starting copies (+) of the β-globin DNA template is plotted.

and 4×10^5 starting copies of template. The plateau region occurs at a higher cycle number and lower signal strength for 4×10^5 starting copies, in agreement with previous work.[32]

A fundamental step in the development of complex microfabricated DNA analysis systems is the demonstration that DNA amplification and analysis can be functionally integrated on a single microdevice. This successful integration is in many ways similar to the fabrication of the DNA analog of an integrated circuit. In this work, we have coupled a microfabricated PCR chamber and

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CE chip and shown that PCR and CE can be directly integrated (1) to perform rapid, hands-off DNA analysis, (2) to amplify targets from genomic DNA, and (3) to analyze PCR amplification in real time.

These integrated PCR−CE microdevices have several clear advantages. Microfabricated PCR chambers perform fast thermal cycling of samples so amplification can be completed in as little as 15 min, and microfabricated CE chips perform electrophoretic analysis of the PCR product in under 120 s; therefore, these integrated devices have the potential to dramatically speed up PCR analysis of targets. The ability to amplify and detect PCR products from genomic DNA, such as *Salmonella*, demonstrates that integrated PCR−CE microdevices have the potential to be used for fast, remote diagnostics. Furthermore, our PCR−CE microdevices are applicable to all types of PCR amplifications, because no complicated or expensive target-specific probe chemistry is required for fluorescence detection of the PCR product, which is distinguished from false amplification by electrophoretic separation. This allows informative real-time monitoring of PCR, with considerably less expensive and less complex instrumentation than that previously used for real-time PCR monitoring.[32] Finally, integration of the PCR amplifier and CE analyzer eliminates the manual transfer of liquid that normally occurs between amplification and separation, thereby simplifying the procedure, eliminating manual pipetting errors, and reducing opportunities for contamination.

PROSPECTS

The good results obtained with the junction we have fabricated here between the PCR and CE devices indicate the great potential for microfabricated integrated DNA analysis systems. A more sophisticated bonding between the devices, such as anodic bonding or polyimide, should lead to a system that is even easier to fabricate and use. Such improvements should also allow the fabrication of arrays of PCR chambers coupled to capillary arrays[6] on chips, which would increase the throughput of these microdevices. In addition, although the "electrophoretic valve" works well in our application, more sophisticated devices with active microfabricated valves such as bistable polymer diaphragms[33] would improve injection efficiency. Simple improvements in the placement of the fan on our integrated microdevice could increase cooling rates to 5 °C/s and thereby decrease the cycle times to below 20 s, so a 30 cycle amplification would require just 10 min. Further reduction in the size of the PCR chamber would allow even faster heating and cooling, eventually making the processivity of the DNA polymerase the limiting factor in the speed of PCR. An ideal PCR−CE microdevice would also integrate a method for spiking the separation of the PCR-amplified mixture with an external standard, thus eliminating any ambiguity caused by variations in migration times. One way to address this issue would be to modify the current layout of the CE device by adding an additional channel for simultaneous electrophoretic loading of a standard DNA ladder. Analogous mixing devices based on electroosmotic flow[25] and electrophoresis[30] have been demonstrated.

In summary, an integrated PCR−CE microdevice has been fabricated and shown to perform amplification and electrophoretic DNA analysis much faster than conventional techniques, with a complete absence of manual sample transfer. The demonstration of a functional integrated PCR−CE microdevice is an important step toward complete integration of DNA analyses on chips. The next challenges will be the integration of sample preparation and detection components on a single microdevice. These microfabricated integrated DNA analysis systems, the DNA analogs of integrated circuits, have the potential to significantly impact the Human Genome Project and molecular biology, just as integrated circuits have revolutionized electronics and computers.

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