TOWARDS AN INTEGRATED MICRODEVICE FOR LIQUID DNA EXTRACTION AND AMPLIFICATION APPLICABLE TO FORENSIC DNA ANALYSIS

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ABSTRACT

Historically, most microfluidic devices for genetic analysis have been fabricated in glass, a substrate that is costly and, if not regenerated properly between analyses, can have problems with cross-contamination. The use of polymeric substrates, such as poly(methylmethacrylate) (PMMA), allows for simplified fabrication with the possibility for inexpensive and disposable microdevices. The work presented in this paper demonstrates the use of plastic microdevices for DNA extraction and amplification of buccal swabs applicable to forensic short tandem repeat (STR) analysis, and represents advancement towards less expensive, integrated, disposable devices.

KEYWORDS: DNA Extraction, PCR, PMMA, Forensic STR Typing

INTRODUCTION

Recent developments in forensic DNA analysis, such as *The DNA Initiative*, have been aimed at providing the means needed to reduce the significant casework backlog, which has increased to over 70,000 cases as of January 1, 2008 [1]. However, the current steps involved with DNA analysis, namely DNA extraction and amplification, create a significant bottleneck that affects the overall time-to-result in human identification. The development of a microfluidic platform that is able to perform one or more of these steps has the potential to decrease overall analysis time, increase throughput and rapidly reduce the backlog. There are several advantages associated with microfluidic devices, including reduced reagent consumption and analysis time, which significantly reduces the cost, and the use of a completely closed system to reduce the incidence of contamination.

THEORY

The most widely-used method for DNA extraction and purification is solid phase extraction (SPE), which typically exploits a silica-based solid phase to reversibly bind DNA. SPE has been successfully adapted for use in microdevices [2], however, flow through the packed beds can be affected by uneven packing of the solid phase and high backpressure. The use of a liquid, enzyme-based extraction method eliminates the need for a solid phase and resolves these issues. Liquid extraction utilizes a neutral proteinase (*Bacillus* sp. EA1) that degrades proteins and nucleases, leaving only the nucleic acids in a PCR compatible buffer system. In addition, liquid extraction provides high quality DNA, which is ready for direct transfer to the polymerase chain reaction (PCR) step, in less time than conventional or microchip SPE (Table 1). Finally, the liquid extraction technique is more amenable to future automation and integration with other DNA analysis techniques.

Table 1: Comparison of the liquid extraction method to microchip-based SPE

	Solid Phase Extraction	Liquid Extraction
Packed Bed	Yes	No
Extraction volume	2-4 µL	100 µL
DNA concentration	~ 1 ng/µL	1-6 ng/µL
Number of Steps	3	1
Loss of DNA possible	Yes	No
Analysis Time	20 mins	6 mins

Conventional PCR typically requires between 2 and 4 hours, but with mixtures of modified polymerases, the amount of time needed for STR amplification has been reduced to as little as 36 minutes using a conventional thermal cycler [3]. Similar enzyme mixtures used in combination with a microfluidic PCR device and a non-contact heating method, such as infrared (IR)-mediated PCR, can reduce the time needed for amplification to under 30 minutes. The IR-PCR method, developed in our laboratory, provides faster heating and cooling rates with glass microdevices than can be achieved in a conventional thermal cycler [4].

Glass has been the most commonly used substrate for microdevice fabrication, although in recent years the trend has shifted to using polymeric substrates for microdevices. The processes associated with fabrication of glass microdevice are laborious, time-consuming, and costly. Plastics substrates, on the other hand, are more inexpensive and can be fabricated using techniques such as hot embossing, injection molding and laser ablation [5, 6]. Poly(methylmethacrylate) (PMMA) has been used extensively as a substrate for microchip electrophoresis and flow-through PCR due to the ease of fabrication and the ability of the surface to be coated with various reagents to alter the electroosmotic flow, but little has been done with PCR in plastic devices for forensic DNA analysis.

EXPERIMENTAL

Device Fabrication

Microfluidic PCR devices were designed in AutoCAD LT® software and fabricated in 1.5 mm thick PMMA by laser ablation using a CO_2 laser system. The PCR chambers were etched using a raster cut (60 % power, 100 % speed followed by 40 % power, 100 % speed), the channels were etched with a low power vector cut (3 % power, 4 % speed)

and the reservoirs cut using a high power vector cut (60 % power, 3% speed). This results in PCR chambers that are ~200 µm deep and ~ 0.6-1 µL in volume.

Etched microchips were bonded using a previously described high temperature, low pressure bonding method [7]. Briefly, chips were cleaned with ethanol to remove any dust created by laser ablation. Unbonded microdevices were sandwiched between two glass plates and placed on a hot plate. Weights (1.6 kg) were applied to produce the 20 kPa of pressure needed to bond the PMMA plates (area of chip = 8 cm^2) and heated at 165 °C for 30 minutes for bonding. The temperature was reduced to 80 °C over the course of 30 minutes and annealed at this temperature for an additional 30 minutes. After bonding, chips were filled with a blue dye and visualized under a stereomicroscope to ensure that the chambers and channels were fluidically sealed (Figure 1).

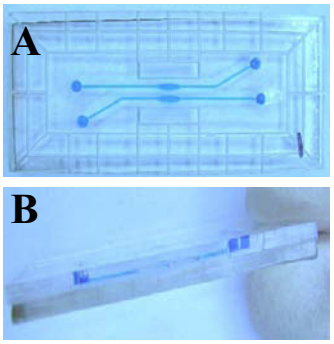

Figure 1: (A) Top view of a PMMA PCR microdevice. (B) Side view of the device. Channels and chambers are filled with a blue dye for better visualization.

Conventional Liquid DNA Extraction and Purification

Buccal swabs were collected from healthy volunteers by swabbing both cheeks with a sterile cotton swab for 30 seconds and allowed to dry overnight. Cells were eluted in 500 µL of deionized water and a 20 µL aliquot was added to the liquid extraction solution, consisting of 10 µL 1X blue buffer, 1 µL forensicGEM™ and 69 µL of water. The sample was incubated at 75 °C for 15 minutes and 95 °C for 5 minutes.

Microchip PCR

After incubation, 2 µL of extracted DNA is added to the PCR master mix, which is comprised of 5 µL Phusion® HF Master Mix, 2.5 µL IdentiFiler® primers, 0.25 µL SpeedSTAR™ DNA Polymerase and 0.25 µL water. The sample is loaded into the PCR microdevice and cycled on the IR-PCR system with the following conditions: 95 °C for 2 min, 32 cycles of 95 °C for 5 sec, 59 °C for 10 sec, 72 °C for 10 sec, and a final extension at 72 °C for 30 sec. Once PCR was complete, the sample was removed from the microchip and prepared for separation and detection using an ABI 310 Genetic Analyzer with GeneScan Software.

RESULTS AND DISCUSSION

One of the main advantages of microdevices in regards to forensic analysis is the ability to have a completely-closed system. This reduces the incidence of contamination by reducing the number of handling and pipetting steps and, with a disposable device, allows for one-time use of the device. The PCR devices described in this work are single-use devices and represent the first steps towards an integrated, disposable device for forensic DNA analysis.

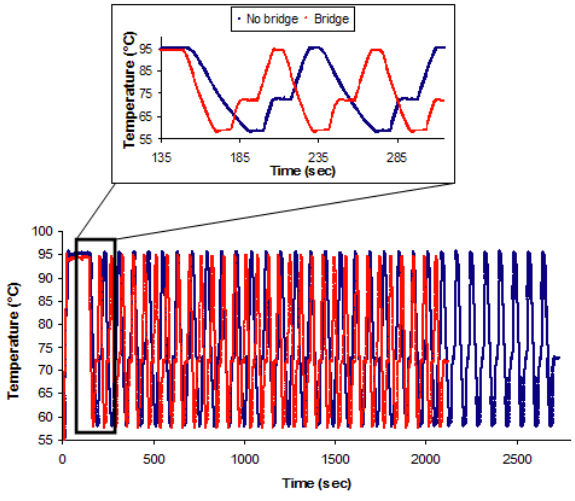

Figure 2: Temperature profiles comparing the bridge and non-bridge chips. Inset - Close-up of the first few thermal cycles after the initial denature.

Using pre-purified DNA, the PCR master mix was optimized for use in PMMA devices by adapting a previously described master mix for use in glass devices [8]. The addition of a coating reagent, such as bovine serum albumin (BSA) or poly(ethylene glycol) (PEG), was found to be beneficial, although not required for effective amplification – a true discriminator from glass. When present, however, it increased the mass of amplicon generated (peak height) in PMMA devices (data not shown). Due to the thickness of the PMMA, slow heating and cooling rates, on the order of 1 °C/s, resulted. To remedy this and increase the heating/cooling rates of the PMMA devices, rectangles were cut on either side of the PCR chambers to reduce the thermal mass [9]. These 'bridged' chips provided significant improvement in the heating/cooling rates and reduced PCR cycling times from 45 minutes in non-bridged chips to 35 minutes in bridged chips (Figure 2). The ramp rates can be increased even further by reducing thermal mass by fabricating devices with thinner PMMA (data not shown).

For the development stages of the integrated device, it was necessary to determine if liquid extraction could be performed in a PMMA device. The extraction solution was prepared as stated above and ~ 6 µL was loaded into the device. Incubation was performed using the IR-PCR system which allowed the incubation time to be reduced to as little as 2 minutes. Once DNA extraction was completed, the sample was removed from the chip, added to a conventional PCR master mix and conventionally thermal cycled. The results yielded a full (16/16 loci) IdentiFiler® profile, which was comparable to the results obtained from a conventional extraction and amplification of the same sample, with a total analysis time of ~1 hour (Figure 3). This is a reduction of at least 3 hours of analysis time when compared to conventional methodologies.

The next phase of development was to perform liquid extraction off-chip and add a sample of the extracted DNA to a microchip PCR master mix to determine if enough DNA from the liquid extraction method would be present in the PCR chamber. To guarantee a high yield and, therefore, a large quantity of DNA for PCR, modification to the liquid

extraction steps were investigated by either adding more of the eluate, increasing the amount of enzyme added and/or simply placing a portion of the dried buccal swab in the liquid extraction solution. Interestingly, it was found that allowing the buccal swab to remain in the extraction solution yielded the highest mass of DNA, however, only a partial (7/16 loci) IdentiFiler® profile resulted (Figure 4), possibly due to dilution of the DNA upon mixing with the master mix and loading onto the microdevice. However, this can be easily remedied by reducing the extraction volume from 100 µL to as little as 25 µL, potentially providing a 4-fold increase in DNA concentration. Furthermore, the amount of enzyme in the reaction can be increased which has been shown to increase DNA yield when the amount of starting cellular material stays the same (data not shown).

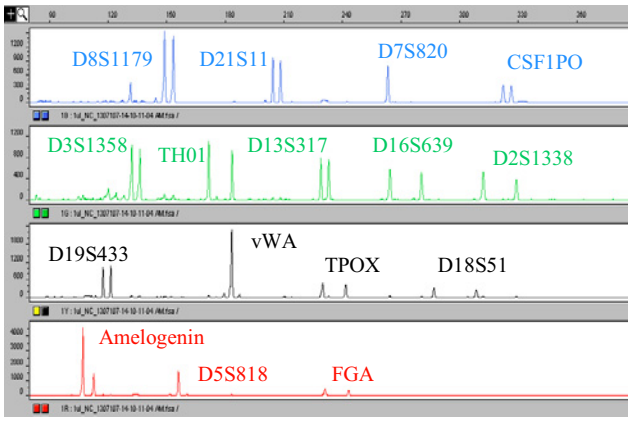

Figure 3: Full IdentiFiler® profile (16/16 loci) achieved after microchip incubation of buccal swab eluate in the liquid extraction solution, followed by conventional PCR.

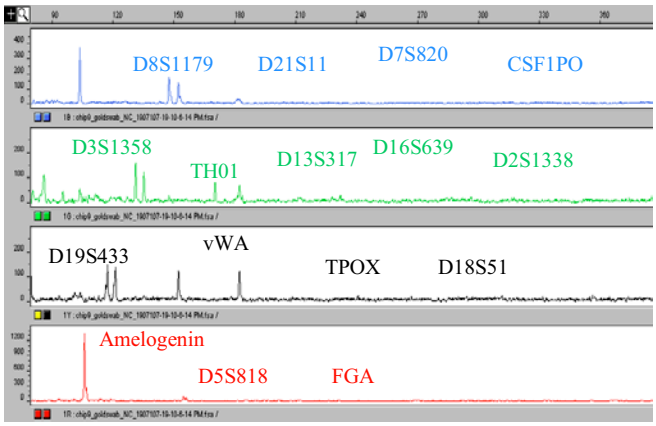

Figure 4: Partial IdentiFiler® profile (7/16 loci) achieved after conventional incubation of a fragment of buccal swab in the liquid extraction solution, followed by microchip PCR.

CONCLUSION

The work presented in this paper describes the initial step towards the development of a plastic, integrated device (Figure 5) for extraction and amplification of DNA from forensic samples, such as a buccal swab or blood stain. The PCR device is capable of a 4-fold reduction in the amount of time necessary to extract, purify and amplify DNA through the use of a liquid extraction method and a PCR master mix containing a mixture of modified polymerases.

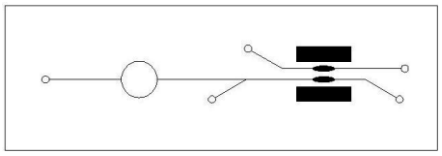

Figure 5: Schematic of an integrated PMMA device for liquid DNA extraction and microchip PCR. Dimensions: 2 cm x 6 cm; liquid extraction chamber - 5 mm diameter

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