HIGH ASPECT RATIO SILICON MICROSTRUCTURES FOR NUCLEIC ACID EXTRACTION

Lee A. Christel, Kurt Petersen, William McMillan, Gregory T. A. Kovacs

Cepheid

1190 Borregas Ave
Sunnyvale, CA 94089

ABSTRACT

Silicon fluidic microchips with high surface area to volume ratios have been fabricated using deep reactive ion etching (DRIE) technology [1,2]. These microchips have been used as capture surfaces for nucleic acids (NA). Short (500 bp) and medium size (48,000 bp) DNA have been captured, washed, and eluted with these chips using glass binding chemistries. DNA quantities approaching 40 ng/cm² of binding area were captured from input solutions in the 100-1000 ng/mL concentration range, and detected using fluorescence techniques. For low concentration studies, PCR and gel electrophoresis were used as the detection method. Extraction efficiencies of about 50%, and concentration factors of about 10 have been demonstrated using bacteriophage lambda DNA as the target. From an input volume of 500 μ L containing 5×10^4 DNA molecules, 2.3×10^4 DNA molecules were extracted and eluted into a 25 μ L volume. These results demonstrate the viability of utilizing such microchips as an element in a compact, disposable cartridge format for the detection of DNA in applications such as clinical diagnostics, biowarfare agent detection, food quality control, and environmental monitoring.

INTRODUCTION

Important diagnostic tests such as HIV viral load in the blood of clinical AIDS patients depend on the detection of small quantities of DNA or RNA. Such tests utilize polymerase chain reaction (PCR) [3] to amplify (multiply) the number of copies of a target sequence to a detectable level. The PCR technique requires a relatively pure DNA sample in aqueous solution, free of contaminants that inhibit the PCR process. Such inhibitors include heavy metals, such as the iron in hemoglobin, nucleases, certain proteins and enzymes found in raw biological samples. There is thus a need for methods to extract and purify DNA from a variety of samples.

Many DNA purification kits are available commercially [4], each for a particular DNA or RNA and input sample. Typically, these kits utilize some form of silica gel, glass matrix, or membrane as the capture medium since it is known that NA will bind to glass or other silica-type surfaces under the proper chemical conditions [5]. A typical protocol for DNA purification involves a number of steps whereby various reagents are added to the sample and the sample is centrifuged to separate precipitated components from the solution. Such a procedure is quite involved, and in many cases is still done manually. Although such a procedure can be automated using robotics, integrating such a protocol into a low cost, disposable analysis format requires a reexamination of the entire process.

As the broad application of the PCR technique continues to be refined and recognized, there is enormous market pressure to improve the cost and throughput of DNA/RNA tests. There are many applications where a small DNA analysis tool utilizing disposable cartridges would expand markets if the costs and throughputs can be improved. Such cartridges would likely combine low-cost plastics technology with selected

micromachined components for critical functions such as DNA extraction. The motivation for this work is the broader goal of developing an automated analysis system which takes raw sample inputs and gives a PCR result in a few minutes. Flow-through cartridge designs capable of reagent containment, mixing and delivery for the processing of significant clinical sample volumes, while maintaining small internal volumes of critical components, appear promising. DNA extraction chips are an important component of this strategy.

We have fabricated high surface area DNA capture surfaces of oxidized silicon using deep reactive ion etching (DRIE) and are investigating the ability of these structures to controllably capture and release DNA from test solutions. Several parameters are of primary importance if such a chip is to be commercially viable. The chip must have sufficient binding capacity to retain the quantity of DNA required for the subsequent assay. It must extract the DNA efficiently, i.e. the ratio of target DNA captured to target DNA input must be high (exceed 50%). It must be able to complete the extraction in a reasonable amount of time, i.e. the capture must occur at reasonable flow rates, so that clinically realistic volumes (sometimes several mL) can be processed expeditiously. The chip must also concentrate the input DNA, allowing the DNA to be eluted (released) into a small volume for PCR amplification. These volumes are typically 100 μ L or less to conserve expensive PCR reagents. The chip must allow wash solutions to be efficiently passed through while retaining the DNA, so that contaminants, such as PCR inhibitors, can be excluded from the final elution without requiring large volumes of wash solution typically associated with commercial kits. Finally, the chip must have a reasonable cost, preferably less than one dollar, in order to be used in a disposable format. If the above conditions can be met, there is a large market opportunity for DNA assays in a wide range of markets, including clinical and veterinary diagnostics, food and environmental monitoring, forensics, and biowarfare detection.

CHIP FABRICATION

The process used to produce the DNA capture chips is shown in Fig. 1. This process is simpler than competing technologies such as LIGA [6]. Fabrication starts with 100mm (100) silicon, double-side polished, approximately 400 μ m thick. The wafer is first oxidized to produce a thin pad oxide, and then coated with silicon nitride. The backside is patterned and etched in KOH to produce fluidic ports that are 200 μ m deep. The frontside is next patterned with the DRIE mask, and etched for approximately one hour in an STS DRIE plasma etcher to produce a field of up to 5,000 pillars, each being 200 μ m high. At the end of this etch, the frontside pattern meets the backside ports. The wafer is further oxidized to coat the internal surfaces with silicon dioxide. The silicon nitride prevents additional oxidation of the top surface. The nitride and underlying pad oxide are now removed by plasma and wet etching to produce a bare silicon upper surface. A cover of pyrexTM is anodically bonded to the wafer to finish the process. The wafer is then ready for sawing to produce the finished square die, which are 3.85 mm on a side.

Figure 1. Process flow for the fabrication of high aspect ratio DNA capture surfaces in silicon

Figure 2 shows an SEM of one chip surface consisting of 200 μm high columns, each about 20 μm in diameter, with a pitch of 34 μm . Chips with pillar diameters of 10 μm and a pitch of 18 μm have also been made. After anodic bonding to the glass top cover, the chip has a total internal surface area of 36 mm^2 in an 'active' area of 3.5 mm^2 .

EXPERIMENTAL DETAILS

The DNA capture studies reported here can be divided into two types: high concentration inputs (on the order of 100-1,000 ng/mL) and low concentration inputs (at or below 10^5 copies of target DNA). These two regimes have relevance to different clinical situations. Samples involving genomic DNA, such as might be present in a sample containing lysed white blood cells, typically have large concentrations of DNA. On the other hand, samples used for diagnosis of infectious diseases often contain only very small amount of pathogenic DNA of interest. Serum, plasma, and urine are examples of the latter situation. For high concentration studies, the quantities of DNA are large enough that fluorescence techniques can be used to detect the DNA. For low concentrations however, PCR must be used to first amplify a target sequence, and only then can the presence of the target DNA be verified usually by gel electrophoresis. Quantitation of the PCR product is more difficult, and the use of calibration standards run through PCR in parallel is mandatory.

Figure 2. SEM of DNA Capture Chip Surface. These pillars are 200 μm high with a pitch of 34 μm .

For DNA capture studies, a fluidic test system has been constructed. It consists of a chip holder, and several computer controlled syringe pumps and valves. A schematic representation of the fluidic system is shown in Fig. 3. An acrylic chip holder allows multiple fluidic connections to be made to the backside of the chip via 0.01" ports and an elastomeric gasket between the chip and fixture. Two syringe pumps allow fluids to be aspirated from source reservoirs into storage loops, and dispensed either to the chip or to waste. Several distribution valves are used to control fluid routing. All valve and pump motions are controlled via the RS-232 interface of an IBM-compatible PC. Protocols are written and stored as stand-alone programs, insuring that the experimental conditions are consistent from run to run.

Starting solutions were obtained by diluting high concentration DNA stock solutions with a glass binding solution from a commercially available DNA purification kit [4]. This binding solution is a chaotropic salt solution, intended to both denature proteins that might be present in a real clinical sample, and to produce conditions that allow binding to glass. Such a solution is a good representation of the chemical environment that would be presented to the chip in a real clinical sample. Wash and elution reagents were also taken from the same commercial kit. The wash solution is typically an ethanol-based solution, with several other ionic components. Elution solutions are either TE (10 mM Tris-HCl and 1 mM EDTA), or water.

A typical experimental protocol is as follows. The chaotropic DNA starting solution is first passed through the chip, during which DNA present in the solution binds to the silicon dioxide-coated microstructure. This is then followed by the ethanol-based wash solution. The wash solution is intended to wash away the salts and other PCR inhibitors that might be present in the sample. Finally an elution reagent is passed through the chip, releasing the DNA back into the fluid stream. This elution is collected in small aliquots for analysis.

For detection of elutions of high concentration DNA, a Carl Zeiss fluorescence microscope was used in conjunction with a photon-counting photomultiplier tube (PMT) and software from Photon Technologies, Inc (Monmouth Junction, NJ). The microscope was fitted with a filter set designed for fluorescein. This filter set allows excitation of the sample at 490 nm, and detection at 515 nm and beyond. This setup is suitable for fluorescein-labeled DNA, as well as for several other DNA dyes, such as YOYO-1 and PicoGreen [7].

Figure 3. Schematic of Fluidic Test System.

For low concentration studies, chip output was processed through a standard PCR protocol using a Perkin Elmer 9700 thermal cycler. Reference samples spanning several orders of magnitude in target starting copy number were run in parallel. PCR product was then processed through gel electrophoresis, typically with 1% agarose gels and ethidium bromide staining. Photographs of the resulting gel bands were compared visually to the reference standards to estimate the starting copy number in the chip elutions. Work in progress is aimed at improving further the quantitation of the PCR product.

HIGH CONCENTRATION RESULTS

For high concentration studies, a solution of plasmid digest DNA from Bio-Rad was used as the starting material [8]. This solution consists of a mix of DNA with an average length of about 500 bp. The material is tagged with fluorescein, a fluorescent marker, allowing in-situ observation of the DNA during sample processing.

The chip elutions were collected into a Delrin fixture consisting of a number of small wells, each with a volume of approximately 25 µL. A number of 20 µL elution droplets were captured into the wells. In parallel, a series of standards of known concentration were dispensed into separate wells. 5 µL of 1.0 µM YOYO-1 dye [7] were then added to each well. This dye further enhances the fluorescence of the samples. After a short incubation

period, the wells were measured for signal using the fluorescence microscope/PMT system.

The above protocol was performed under several experimental conditions. The ranges of experimental parameters are given in Table 1. Note that the internal volume of the chip is about 0.2 µL, leading to a residence time of 200 msec at a flow rate of 1.0 µL/s.

Table 1. Experimental Conditions for High Concentration DNA Capture Experiments.

Parameter	Range
DNA Conc.	100 - 1000 ng/mL
DNA Volume	400 µL
DNA Total Dose	40 - 400 ng
DNA Flow Rate	0.1 - 5.0 µL/s
Wash Volume	400 µL
Wash Flow Rate	0.5 - 5.0 µL/s
Elution Flow Rate	0.5 µL/s

The total binding capacity of high aspect ratio chips was investigated using an input of 400 ng of DNA (400 µL of a 1,000 ng/mL solution) followed by 400 µL of wash and then elution. All flow rates were 0.5 µL/s. The fluorescence signals from the sample wells, as well as those of the reference standards are shown in Fig. 4. By comparing each elution signal to the standards, and summing them, we conclude that 11-12 ng of DNA has been captured, then eluted from the chip. It has been reported [9] that the maximum binding capacity of glass is ≈ 40 ng/cm². Since the internal surface area of the chip is ≈ 0.36 cm², it is evident that in this experiment, an amount of DNA consistent with the maximum binding capacity of glass has been captured.

As a comparison, the same protocol was run utilizing a 'flat' chip without the enhanced surface area. This chip had an internal surface area of about 0.06 cm². In this case, about 2.5 ng of DNA was captured and eluted, very nearly 1/6 of that captured on the DRIE chip. This is also consistent with binding at the capacity limit of glass.

Figure 4. Fluorescent signals from DNA chip elutions, with reference samples. The total DNA captured is about 12 ng.

Several other experimental conditions were examined. It was found that DNA flow rates higher than 0.5 µL/s led to reduced capture efficiency, but wash flow rates could be increased up to 5.0 µL/s without loss of efficiency. These results are summarized in Table 2.

Table 2. Experimental Conditions for High Concentration DNA Capture Experiments. All input volumes were 400 μ L.

DNA Conc (ng/mL)	DNA Flow Rate (μ L/s)	Wash Flow Rate (μ L/s)	Capture Efficiency (%)
1000	0.5	5.0	2.8 ¹
100	0.1	0.5	10
100	0.5	0.5	10
100	0.5	5.0	10
100	5.0	5.0	5

¹ DNA capture is at the binding limit of the structure.

LOW CONCENTRATION RESULTS

For the low concentration studies, lambda DNA (48,000 bp) [10] was used as a bacteria simulant. Starting solutions were again prepared by dilution of lambda stock with chaotropic salt solutions. Starting copy number for the first set of experiments was 5×10^4 copies in 500 μ L of solution. As a control, the experimental protocol was first run using a chaotropic solution without DNA. Standards of known copy number (10^4 and 5×10^4) were also prepared using both water that had passed through the chip test system, and pure water.

The protocol was similar to that of the high concentration studies. Typically, 500 μ L of the input test solution was passed through the chip at 0.5 μ L/s, followed by 250 μ L of wash solution. Following the wash, an air gap was introduced to delineate the wash/elution boundary. Elution agent was introduced into the chip, and allowed to stand for several minutes. Elution droplets were then collected directly into PCR reaction tubes. Four elution samples were taken from the chip, consisting of three samples of 25 μ L followed by an additional sample of 50 μ L. The 25 μ L samples were diluted up to 50 μ L after collection so that all samples submitted to PCR were 50 μ L. The 500 bp PCR products were run through gel electrophoresis and photographs of the gel (under UV illumination) were taken and used to compare the chip elution samples to the standards of known copy number.

Fig. 5 shows the gel photograph from one such experiment. The standards prepared with system water are shown first, followed by the run with blank solution, the run with DNA solution, and standards prepared with pure water. By comparing the first 25 μ L chip elution to the 10^4 and 5×10^4 standards in system water, it is estimated that the first chip elution contains about 2.5×10^4 copies. This implies a 10X concentration effect, and a 50% capture efficiency. There is a small amount of PCR inhibition evidenced by the fact that the 5×10^4 standard prepared using system water is not as intensely stained as the 5×10^4 standard prepared using pure water.

CONCLUSIONS

DNA analysis technology is playing an increasing role in many health-related areas. Laborious, manual procedures introduce human variability, possible cross contamination and high cost. By replacing such procedures with automated, disposable assays, the cost and applicability of this exciting new technology will gain the widest possible market. Automated bench-top and hand-held DNA analysis tools utilizing disposable cartridge formats are being developed to serve this need. As a component of such systems, the use of silicon microstructures for the extraction and concentration of target DNA can play an important role.

Figure 5. Photograph of electrophoretic gel showing DNA chip capture. Lanes: M: Molecular weight standard, 1: Neg Ctl, 2: 10^4 standard in system water, 3: 5×10^4 standard in system water, 4-6: control run with no DNA, 7-10: Chip Elutions from DNA run, 11: 10^4 standard in pure water, 12: 5×10^4 standard in pure water. By comparing lane 7, obtained from the first 25 μ L chip elution, to standard lanes 2 and 3, it is estimated that a capture efficiency of 50% was achieved, with a concentration factor of about 10X in the first elution.

We have shown that deep reactive ion etching can be used to produce high surface area structures suitable for use as DNA capture elements. With proper design of structure and protocol, we believe such devices will be critical, cost effective components in future DNA analysis systems.

ACKNOWLEDGMENTS

The authors would like to thank Rick Faeth for wafer fabrication, and Linda Western and Sandy Zaner for PCR processing. This work was supported in part by funds from the United States Army Medical Research Institute of Infectious Diseases (USAMRIID).

REFERENCES

1. J.K. Bhardwaj, H. Ashraf, "Advanced Silicon Etching using High Density Plasmas", *SPIE Micromachining and Fabrication Technology Proc.* 2639, pp. 224-233 (1995).
2. B. P. van Drinhuizen, N. I. Maluf, I. E. Opris, and G. T. A. Kovacs, "Force-Balanced Accelerometer with mG Resolution, Fabricated using Silicon Fusion Bonding and Deep Reactive Ion Etching", Technical Digest of the 1997 International Conference on Solid-State Sensors and Actuators, Transducers '97, Chicago, IL, pp. 1229-1230 (1997).
3. R.K. Saiki, S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlick and N. Arnheim, "Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia", *Science* 230, pp 1350-1354 (1985).
4. Biorad Labs, Hercules CA, Promega, Madison WI, and Qiagen Inc., Santa Clarita, CA.
5. Willem R. Boom et al, US Patent 5,234,809 (Aug 1993).
6. Christophe Marques, Yohannes M. Desta, James Rogers, Michael C. Murphy, and Kevin Kelly, "Fabrication of High-Aspect-Ratio Microstructures on Planar and Nonplanar Surfaces Using a Modified LIGA Process", *JMEMS* 6(4), pp. 329-336 (1997).
7. Molecular Probes, Eugene OR. YOYO-1: P/N Y-3601, PicoGreen: P/N P7581.
8. Bio-Rad Low Range Fluorescein Standard P/N 170-3123.
9. Bert Vogelstein and David Gillespie, "Preparative and analytical purification of DNA from agarose", *Proc. Natl. Acad. Sci.*, 76(2), pp. 615-619 (1979).
10. Pharmacia, Bridgewater, NJ.