Microfabricated systems applied for DNA amplification and for flow cytometry

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Microfabricated systems applied for DNA amplification and for flow cytometry

By

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Preface

This thesis is written as a requirement for obtaining the Ph.D. degree at the Technical University of Denmark (DTU). The Ph.D. project took place at Mikroelektronik Centret (MIC), DTU, from March 2000 to May 2003.

The Ph.D. project was conducted within the Cell Particle Handling project and was supervised by project leader, Dr. Anders Wolff (main supervisor) and the Director of MIC, Prof. Pieter Telleman (co-supervisor). The project was funded by 'Statens Teknisk Videnskabelige Forskningsråd' (STVF) and performed under the Chemistry and Biotechnology Ph.D. program at DTU.

Background

The Cell Particle Handling project originated from Anders Wolff in an attempt to create a microfabricated total analysis system which was able to sort cells, amplify DNA from a specific cell type, and perhaps also analyze the amplified DNA. The cell sorting method should be provided by dielectrophoresis (DEP) and the DNA amplification should be performed by polymerase chain reaction (PCR). The DNA analysis method was to be decided at a later point, as it might not be integrated in the first version of the cell sorting and amplification device.

The project

To speed up the development of the integrated device, it was decided to split up the DEP and the PCR into two separate devices, which would then be integrated again at a later phase. This Ph.D. project describes the development of the microfabricated PCR chip, including some of the challenges this work resulted in. It also includes some sections concerning the design of the PCR chip, as well as the application of DEP as sample pretreatment for PCR. During visits to the Laser Spectroscopy and Chemical Microtechnology Group at Oak Ridge National Laboratory, Tennessee, US, I was introduced to micro flow cytometry. This resulted in a study of applying a cytotoxicity assay on-chip. This work is also included in the present Ph.D. thesis.

Acknowledgements

Several people have to varying degrees been involved in this project. First of all, I would like to thank my supervisor, Anders Wolff, for his enthuasiasm and collaboration during this project. I

would also like to thank my co-supervisor Pieter Telleman, for his advice on various topics relating to the project.

I have appreciated the friendship of my fellow group members Jamil El-Ali and Ivan R. Perch-Nielsen. Their support has kept the spirit high throughout the project. Without their collaboration and expertise this project would not have been possible.

A special thanks goes to the Laser Spectroscopy and Chemical Microtechnology Group at Oak Ridge National Laboratory. All the group members made me feel welcome during my external research visits and I appreciate that. I would especially like to thank Stephen C. Jacobson, Christopher T. Culbertson, and J. Michael Ramsey for inviting me to visit the group in the first place and for their help with the flow cytometry work.

I am grateful to with Heidi Dvinge, special course student, for her collaboration in the work on dynamic coating with BSA and salmon DNA in chip structures. Others at MIC I would like to thank include the Bioarray group for valuable input and for letting me occupy their equipment for an extended period of time. It has been a privilege to work at MIC. The changes in ‘processen’ have made the spirit at MIC special, unlike any other university institute.

Finally, a special thank you goes to my family for their moral support and patience throughout this Ph.D. project, especially Jo and Thomas Riber, who have put a lot of effort into proof reading and editorial work on the figures.

Kongens Lyngby, May 2003.

Claus Riber Poulsen

Abstract

Since the early 1990's, the 'miniaturized total chemical analysis systems' (μTAS) concept, also known as 'Lab on Chip' has evolved considerably. Sampling, detection, and read-out steps in a μTAS are ideally performed on a single microchip.

The microchip format offers several benefits. These benefits can be utilized in biological applications of microfabricated analysis systems. In this thesis, applications for flow cytometry and polymerase chain reaction (PCR) are presented.

Flow cytometry is used for the development of an acute cytotoxicity assay. A micromachined chip makes it possible to mix reagents and cells on-chip. Therefore, a cellular response can be observed from start to finish with fast acting reagents. The 25 cm detection channel makes on-chip dynamic assays possible. Results from the developed assay indicates that the Triton X-100 effect on cell membranes sets in between 0.2 and 25 seconds, and has not yet reached a steady state after 47 seconds.

PCR is performed in a SU-8 based reaction chamber fabricated on a glass PCR chip. The chip has integrated thin film heaters and temperature sensor electrodes. In this design heating and cooling rates of 50°C/s and 30°C/s respectively have been obtained. It was required to passivate the SU-8 surface before PCR was possible to perform. However, after silanization an average PCR yield of 68% compared to standard PCR tubes was obtained.

A practical use for the PCR chip was demonstrated by *Campylobacter jejuni* detection. The PCR chip proved successful in amplification of DNA from various sources. It was even possible to perform PCR directly on whole *Campylobacter jejuni* cells. The detection time has decreased significantly with the chip detection method, compared to conventional methods. The chip method can be performed in less than a day, where just under a week is needed for conventional detection methods.

Finally, dielectrophoresis (DEP) is demonstrated as a sample pretreatment that can be integrated with PCR. In the presented example yeast cells were separated from the known PCR inhibitors haemoglobin or heparin. Subsequent PCR was only successfully if performed on cells treated in DEP chip. DEP microsystems is here demonstrated as an effective sample pretreatment for PCR.

Resumé

Siden starten af 1990'erne er konceptet omkring miniaturiserede Total Analyse Systemer (μTAS) udviklet betydeligt. Visionen bag μTAS er at formindske alle laboratorium analysetrin til et mikrochipformat. Derfor er μTAS også kendt som 'Lab on Chip'. Analysetrin som prøveopsamling, detektion, samt signalbehandling foregår ideelt set på én mikrochip i μTAS. Dimensionerne som mikrochipformatet tilbyder, giver μTAS adskillige fordele. Disse kan udnyttes ved biologiske anvendelser af mikrofabrikerede analysesystemer. I nærværende afhandling er anvendelser af flow cytometri og polymerase kædereaktionen (polymerase chain reaction, PCR) beskrevet, samt demonstreret i mikrofabrikerede systemer.

Flow cytometri er her benyttet i udviklingen af en akut cytotoksisitetstest. Den fremstillede mikrochip muliggør blanding af reagenser og celler i chippen. Herved kan cellers respons følges præcist fra forsøgsstart til slut på hurtigtvirkende reagenser. Da mikrochippen har en 25 cm detektionskanal er dynamiske reaktionsanalyser mulige. Det demonstreres, at virkningen af Triton X-100 på cellerne starter i tidsperioden 0,2 til 25 sekunder, men ikke er ophørt efter 47 sekunder.

I afhandlingen udføres PCR i et SU-8 baseret reaktionskammer på en mikrochip af glas. Chippen har integrerede varmetråde og temperaturfølere, der muliggør en god temperaturkontrol samt køle-varmerater på henholdsvis 30°C/s og 50 °C/s. Inden PCR i en chip var mulig, var det nødvendigt at inaktivere SU-8 overfladen ved silanisering samt vask. Herefter blev et gennemsnitlig DNA udbytte på 68% i forhold til standard PCR rør opnået.

Som praktisk eksempel på anvendelsen af PCR chippen blev detektion af *Campylobacter jejuni* demonstreret. Alle undersøgte DNA prøver var vellykkede på PCR chippen. Yderligere kunne hele *Campylobacter jejuni* celler bruges direkte til PCR. Den samlede detektionstid blev nedsat betydeligt ved brug af PCR chip detektionsmetoden. PCR chip detektionsmetoden tager omkring en dag, mens standard detektionsmetoden tager omkring en uge alt inklusiv.

Til slut er dielektroforese (DEP) som prøveforberedelse for PCR præsenteret. Denne mikrochip-baserede metode blev anvendt ved adskillelse af gærceller og PCR-hæmmerne heparin eller hæmoglobin. PCR var udelukkende vellykket efter separation i DEP-mikrosystemet af celler og PCR-hæmmere.

Publications

Refereed papers in journals and proceedings

Wolff, A., Perch-Nielsen, I.R., Larsen, U.D., Friis, P., Goranovic, G., **Poulsen**, **C.R.**, Kutter, J., Telleman, P. Integrating advanced functionality in a microfabricated high-throughput fluorescent-activated cell sorter. *Lab-on-a-chip* 2003, **3** (1), 22-27.

El-Ali, J., Perch-Nielsen, I.R., **Poulsen**, **C.R.**, Jensen, M., Telleman, P., Wolff, A. Microfabricated DNA amplification device monolithically integrated with advanced sample pre-treatment. *Accepted for oral presentation at Transducers '03, Boston, USA.*

El-Ali, J., Perch-Nielsen, I.R., **Poulsen**, **C.R.**, Telleman, P., Wolff, A. SU-8 based PCR chip with integrated heaters and thermometer. *Eurosensor XVI, Prauge, Czech Republic, September 2002, pp. 277-278 and CD-rom p502-504.*

Perch-Nielsen, I.R., **Poulsen, C.R.**, El-Ali, J., Bang, D.D., Wolff, A. Removal of PCR inhibitors using dielectrophoresis as a selective filter in a microsystem. *Submitted to Lab On a Chip*.

El-Ali, J., Perch-Nielsen, I.R., **Poulsen**, **C.R.**, Bang, D.D., Telleman, P., Wolff, A. Simulation and experimental validation of a SU-8 based PCR thermocycler chip with integrated heaters and temperature sensor. *Submitted to Sensors and Actuators in September 2002.*

Papers in preparation

Poulsen, C.R., Culbertson, C.T., Jacobson, S.C., Ramsey, J.M. Micro flow cytometer for on chip cytotoxicity assay. *Manuscript in preparation*.

Poulsen, C.R., El-Ali, J., Perch-Nielsen, I.R., Bang, D.D., Wolff, A. Detection of a putative virulence *cad*F gene of *Campylobacter jejuni* isolates from different sources using a microfabricated PCR chip. *Manuscript in preparation*.

Ph.D. thesis

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Mikroelektronik Centret – MIC, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark. *Handed in Medio May 2003*.

Abbreviations

μTAS: Micro Tool Analysis System

DNA: Deoxyribonucleic acid

PCR: Polymerase Chain Reaction

SVR: Surface to Volume Ratio

Taq: DNA polymerase from the thermophile bacteria *Thermus aquaticus*.

CE: Capillary Electrophoresis

SiO_2: Silicondioxide

HMDS: Hexamethyldisilazane

PDMS: PolyDiMethylSiloxane

PEG: Polyethylene glycol

PVP: Polyvinyl pyrrolidone

dNTP: Deoxynucleotide

bp: Base pair

DEP: Dielectrophoresis

rTth: DNA polymerase from the thermophile bacteria *Thermus thermophilus*.

SU-8: Polymer compound. Also used in IC fabrication as photoresist.

CONTENTS

1 Introduction

In the early 1990's originated the concept of 'miniaturized total chemical analysis systems' (μTAS) (Manz, Graber et al. 1990). μTAS is also known as 'Lab on a chip'. The concept behind these terms is to shrink an entire laboratory to microchip size. In a true μTAS every analysis step from sampling to detection and signal readout is performed on a microchip (Figure 1). μTAS was originally a mean to enhance analytical performance in analytical chemistry (Manz, Graber et al. 1990), since chemical sensors at the time did not provide the best selectivity and lifetime (Reyes, Iossifidis et al. 2002).

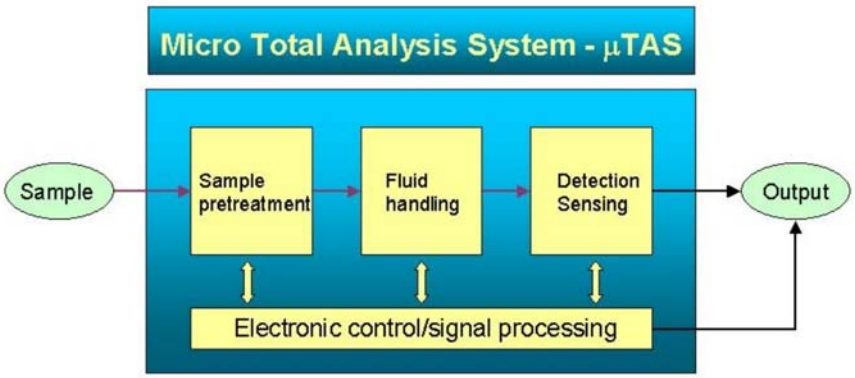

Figure 1. Schematic illustration of the μTAS principle. In a true μTAS every analysis step from sampling to detection and signal readout is performed on microchip. Adapted from (van den Berg and Lammerink 1998).

Over the time several statements about μTAS have been put forward. These statements have included that μTAS will lead to small instruments, integration of various functions, and in the end will facilitate automation. Within the scientific community there are differences of opinion about what the primary advantage of μTAS is (Kricka 1998; Ramsey 1999; McGlennen 2001; Huang, Mather et al. 2002). These differences of opinion are not surprising, since there is not just one single primary advantage. The primary advantage will depend on the task. However, it is generally agreed that the integration of different functions in a single, monolithic design is a major benefit of μTAS, as new functionalities can be developed. Another common agreement is that automation of microfabricated devices is important (Ramsey 1999; Huang, Mather et al. 2002; Verpoorte 2002). Automation improves the efficiency and reproducibility of the laboratory work. The time normally spent on an assay can be used elsewhere, once an automated assay is implemented. In addition, automation of a process also means that novices can operate the machine, and therefore perform

difficult and complex assays in the same way as computers permit the use of advanced tools, without understanding the underlying complex theory (Ramsey 1999). Once an entire analytical process is automated, the true potential of μTAS can be realized in a point-of-care device. The idea behind the point-of-care apparatus originates from the notion of having small, integrated, and automated analytical microchips, which are the base of portable or ideally handheld machines. The analytical devices are designed to move diagnostic testing out of central laboratories into sites closer to patients where the care is needed - 'the point-of-care' (Tüdos, Besselink et al. 2002).

1.1 Flow cytometry

Cell based assays are widely used to study genes, cellular pathways, cell to cell interactions, or drug target investigations (Kroesen, Mesander et al. 1992; Nolan and Sklar 1998; Hesley, Daijo et al. 2002). Today, where the human genome and the genome of several other organisms are fully sequenced, research is moving towards the post genomic era. The next logical step will be to investigate gene-gene or gene-protein interactions, and what effect these interactions have on living cells (Herzenberg, Parks et al. 2002). It will therefore be increasingly important to monitor single cells or cell populations for specific characteristics. Flow cytometry is a rapid screening technique for cell populations on a single cell level.

1.1.1 Flow cytometry in microsystems

By implementing micro machining in flow cytometry, it will be possible to monitor single cells more precisely. This is due to the fact that dimensions in the microsystem are in the same order of magnitude as the studied cells. This improved dimension proportion causes the resolution to be superior to conventional systems. The high quality data that is obtained in single cell observations makes it feasible to lower the number of cells necessary for an experiment. Depending on the data quality, the number of cells per experiment can be as low as 150 cells (Farinas, Chow et al. 2001). This low number widens the usability of flow cytometry to include low volume samples, which contain cells that are either low in concentration or not possible to culture. An example of this is primary cell cultures, as the numbers of cells are few in a dissected sample and the cells are impossible to culture (Farinas, Chow et al. 2001).

There are, however, disadvantages originating from the small size of the device. If a

microfabricated system is not properly attended to, it will quickly be blocked by cell aggregates or debris that stick to the channel walls. This has been solved in different ways. Fu and colleagues (1999) have solved the potential problem by using a silicone elastomer to build the structure. This way the structure is disposable, and laborious cleaning of the microchip is not necessary. McClain and colleagues (2001) used another approach. They treated the channel walls by poly(dimethylacrylamide) to prevent cells or debris from sticking in the channels.

The standard flow cytometry is a pressure driven system (Shapiro 1995), but in microfabricated flow cytometers other driving forces have been investigated (Fu, Chou et al. 2002). One example of a driving force is partial vacuum. Farinas and colleagues successfully used partial vacuum to measure the membrane potential in premonocytic cells (Farinas, Chow et al. 2001). Electrophoretic force has also been used for driving fluid through a micro flow cytometer (McClain, Culbertson et al. 2001). Finally, Fu and colleagues have used the electro-osmotic force in their study to sort labeled *Escherichia coli* cells from non labeled in a μFACS system (Fu, Spence et al. 1999).

The flow cytometry technique in microsystems has the potential of performing various cellular assays. In Chapter 2 is described an acute toxicity assay of cells performed on a microfluidic device, which allows mixing of reagent and cells on-chip. The presented system also shows that surface treatment as illustrated in McClain's study is very useful for preventing cells sticking in channels (McClain, Culbertson et al. 2001), and high data quality can be obtained from a few thousand cells.

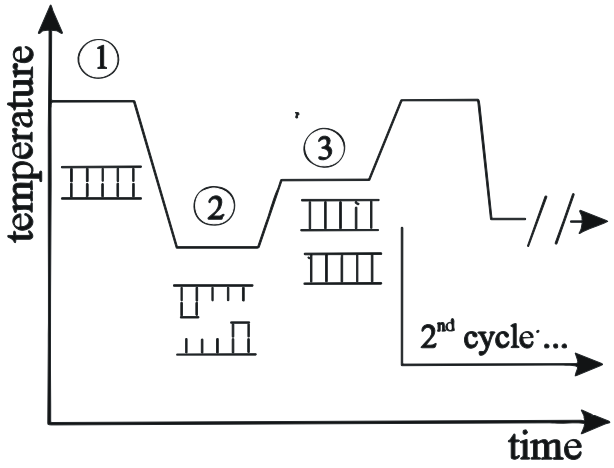

Figure 2. Schematic diagram of PCR amplification. 1 indicate the denaturation step in the PCR, 2 indicate the annealing step, and 3 indicate the extension step in the amplification. One PCR cycle consists of steps 1 to 3 (Schneegass and Kohler 2001).

1.2 PCR

The realisation of polymerase chain reaction (PCR) has had a profound impact on molecular biology. PCR has accelerated the studies of the genetic structure of a diversity of organisms (de Mello 2001). Polymerase chain reaction is an enzyme catalysed amplification that allows any nucleic acid sequence to be amplified in abundance (Mullis, Faloona et al. 1986; Saiki, Gelfand et al. 1988). This happens in a three step process, where the first step is denaturation of the double stranded DNA (Figure 2). The next step in PCR is annealing of primers to the single stranded DNA molecules. The primers are synthetically manufactured oligonucleotides designed to target a specific sequence in each of the two DNA molecules. The pair of oligonucleotides will attach at a very specific site on each DNA strand as the temperature is lowered from the denaturing temperature at about 92°C to annealing temperaure, which typically is about 50°C.

The last step in a PCR cycle is extension of the DNA strand from the annealed primers. This step is performed at a temperature around 72°C, where the enzyme, DNA polymerase, is most efficient. At the extension step, the actual copying happens as the polymerase enzyme constructs a new DNA strand. This happens by incorporating free nucleic bases from PCR solution to the flanking primers, using the original DNA strand as a template. Hence the name template DNA.

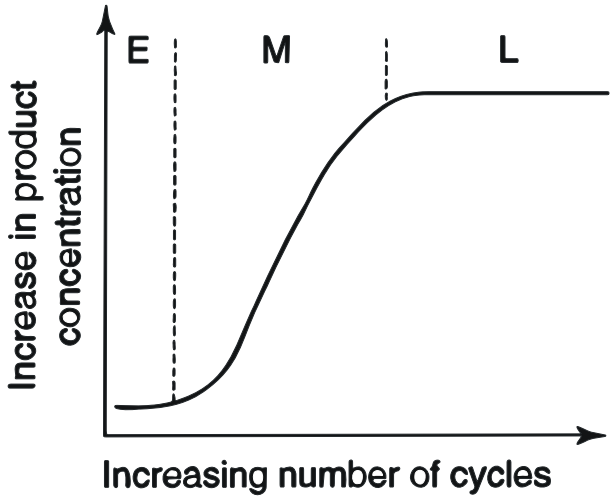

Figure 3. Illustration of DNA target growth in PCR process. E is the early phase of PCR process, M is the mid phase where the number of DNA target grows in an exponential manner, and L is the late phase where the growth levels off (McPherson and Møller 2001).

The newly synthesized double stranded DNA can be melted at the denaturation step, and another PCR cycle can start over again. This cycling is typically performed 25 to 35 times (Huang, Mather et al. 2002). As the number of DNA molecules theoretically is doubled by each cycle, the number of DNA molecules will reach about a million after 20 cycles (McPherson and Møller 2001). Figure 3 depicts the theoretical increase in PCR product in the early, middle, and late phases of PCR. However, amplification is never truly exponential as poor template-primer hybridisation, inefficient thermal cycling, and DNA polymerase inefficiency can play a role in the amplification of the DNA molecules (de Mello 2001).

Numerous applications have been found for PCR within molecular biology such as cloning, genetic engineering, and sequencing (Dieffenbach and Dveksler 1995; Old and Primrose 1996; McPherson and Møller 2001). However, in the most basic form, PCR serves to copy template DNA to levels where it can be detected more easily, and as such the use of PCR within sample pretreatment is beyond dispute (de Mello and Beard 2003).

1.2.1 PCR microchip device

After capillary electrophoresis (CE), PCR is the most investigated DNA analysis method for on-chip use (Beebe, Mensing et al. 2002). A reason for this could be that devices for performing PCR on-chip benefit from several of the advantages that is said about μTAS. The PCR chips have taken

advantage of the low volume, which implies that heating and cooling of the system can be performed faster than in conventional equipment. Furthermore, the mechanistic simplicity of PCR and its dependence on the strict control of experimental conditions, makes PCR ideal to a miniature format (de Mello and Beard 2003).

The first microfabricated device for performing amplification of DNA was reported only a decade ago (Northrup, Ching et al. 1993). Since then numerous structures have been produced both as stand-alone systems and as part of more complex integrated devices (Cheng, Waters et al. 1998; Khandurina, McKnight et al. 2000; Lagally, Medintz et al. 2001). In most microfabricated structures, PCR is used to raise the concentration of the target sequence to a detectable level. However, the use of PCR chips for multiplex amplification are also reported (Cheng, Waters et al. 1998; Waters, Jacobson et al. 1998; Belgrader, Young et al. 2001). By using several primer pairs it is possible to amplify multiple target sequences in one experiment. Another use of amplification on-chip is continuous flow PCR. Kopp and colleagues (1998) first reported this high throughput PCR, but other studies using this method have followed (Schneegass, Brautigam et al. 2001; Sun, Yamaguchi et al. 2002). Quite recently, continuous flow PCR has been combined with reverse transcription where RNA is transcribed into DNA, and afterwards amplified like normal PCR (Obeid, Christopoulos et al. 2003).

Most microfabricated systems are not optimized as well as standard PCR tubes. Therefore, a higher polymerase concentration is needed in these systems. The studies mentioned in this chapter have used DNA polymerase concentrations ranging from 0,025 U/μl to 0,25 U/μl (Kopp, de Mello et al. 1998; Khandurina, McKnight et al. 2000).

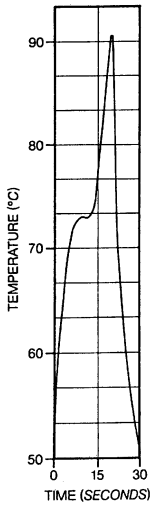

Figure 4. Temperature profile during a 30 second PCR cycle for a 10 μl sample (Wittwer, Fillmore et al. 1990).

One of the objectives for performing PCR on a microscale is to decrease the total amplification time. Reducing the number of cycles in PCR can decrease the amplification time. This solution is not exclusively for application on microchips, but can also be applied to tubes. Another way to decrease amplification time is to shorten the time for temperature cycles. Wittwer and colleagues addressed this topic in a study where the reaction chamber was a capillary tube and hot air provided the heat (Wittwer, Fillmore et al. 1990). This setup has a very low thermal mass, making fast transition times between temperatures possible. The fast transition time is key to the short cycle times obtainable on-chip. In the study, it was found that DNA denaturation and primer annealing occurred almost instantaneously, as amplification was positive when time at denaturation and annealing temperatures were reduced to milliseconds (Figure 4). The investigation showed it was possible to have a total of 30 amplification cycles in as little as 10 minutes, which is 20 s/cycle. In summary, this investigation shows that decreasing the total amplification time indeed is possible for PCR in a microformat.

To accomplish the goal of a fast, miniaturized analysis system, aspects such as heating efficiency and materials have to be considered. These aspects are considered in the next two sections.

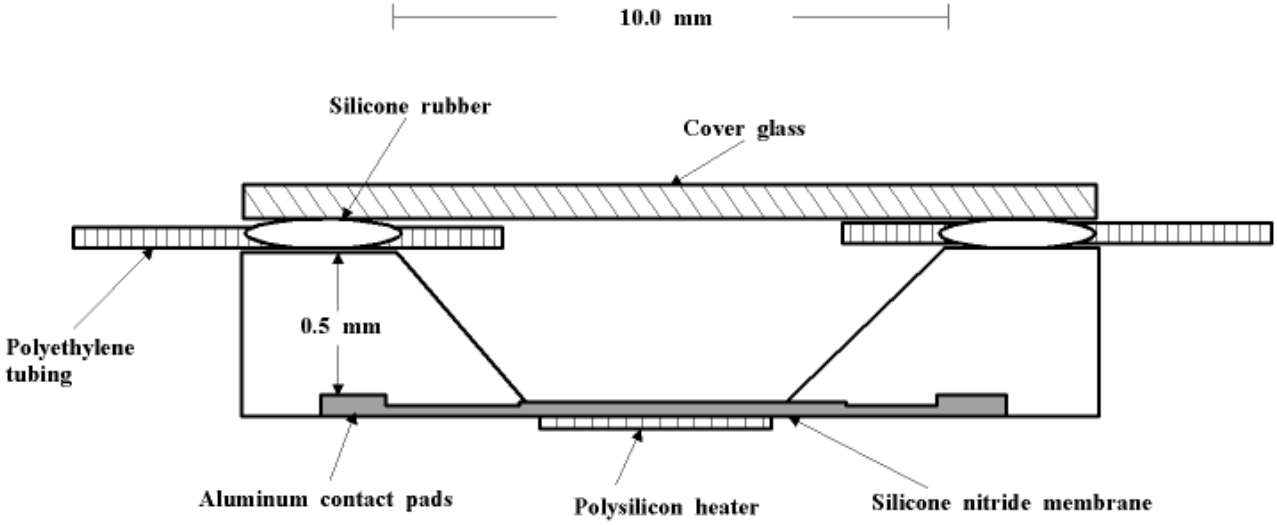

Figure 5. Cross section of the first micro PCR chip device. Polysilicon heaters are integrated under silicon nitride membrane. The polyethylene tubing provide the entry and outlet of the device (Northrup, Ching et al. 1993).

1.2.2 Heating of PCR chip

Polysilicon heaters integrated beneath a silicon nitride surface provided the heating of the first PCR chip (Figure 5; (Northrup, Ching et al. 1993)). In later studies, integrated resistive heaters were also applied on micro fabricated PCR chips (Lee, Hsing et al. 2000; Lagally, Medintz et al. 2001). Integrated heaters have the advantage of an almost direct contact between the PCR solution and the heaters. Therefore, the heat is almost instantly transferred to the PCR solution. Another common thermal source for PCR on-chip is Peltier elements attached to the chip. This heating method cannot provide as fast heating and cooling as integrated heaters. The reason for this is that a Peltier element in itself represents a relatively large thermal mass, compared to the volume of the PCR solution. The Peltier heating method has been applied to substrates of glass (Khandurina, McKnight et al. 2000), silicon (Taylor, Winn-Deen et al. 1997; Wilding, Kricka et al. 1998), and PDMS (Hong, Fujii et al. 2001). Infrared (IR) heating has also been used for PCR with tungsten lamps as the heat source. This has been applied both to PCR experiments in capillaries (Oda, Strausbauch et al. 1998; Huhmer and Landers 2000) and in microchips (Giordano, Ferrance et al. 2001). Heating using IR light happens as water absorbs the energy from the light, which results in bulk heating of the solution. Reaction containers for thermo cycling using IR light have to be transparent for wavelengths in the IR range in order to get a selective heating of the PCR solution. When using IR heating, the container is only heated indirectly by the hot solution inside (Giordano, Ferrance et al. 2001). Recently microwaves have also been reported as a possible

heating source (Fermer, Nilsson et al. 2003). Like IR heating, the microwaves selectively heat the liquid. Due to the very low thermal masses in PCR systems heated by IR light or microwaves, heating and cooling rates can be increased by magnitudes in comparison to standard systems. The low thermal masses make it possible to perform PCR with cycle times that are significantly decreased.

1.2.3 Alternative materials for PCR chips

The same fabrication methods used in production of integrated circuits, e.g. computer chips, are used for silicon-based microchips for biological assays. These fabrication methods require trained staff and special production facilities, and are consequently expensive. Alternative materials and fabrication methods have therefore been investigated for manufacturing PCR microchips

One material that has been examined is the plastic polyimide (Giordano, Ferrance et al. 2001). This type of microchip is manufactured by laser ablation. Even though laser ablation does not involve a special production facility, it does require access to laser apparatus with all the necessary equipment for laser ablation. Another approach is casting of microstructures, which have been accomplished in the polymer polydimethylsiloxane (PDMS) (Hong, Fujii et al. 2001). This method only involves a master mould, which can be used an infinite number of times. PDMS consists of a prepolymer and a curing agent when mixed and cured is making a silicone-like compound. This method has the advantage that only a very limited number of tools are required.

A novel approach for fabrication of a PCR chip is the use of the epoxy-based photoresist SU-8 (Chapter 4, **(El-Ali 2003)**). The advantage of a SU-8 based chip is simple fabrication by standard photolithographic techniques. This will also help in integration with other devices like flow channels. In fact, such an integrated device has already been designed and fabricated (El-Ali, Perch-Nielsen et al. 2003; Perch-Nielsen 2003), although the use of it remains to be tested thoroughly. The use of the photoresist SU-8 is a simple way to produce biocompatible microchips as it only includes a couple of production steps and therefore can be finished in a few days.

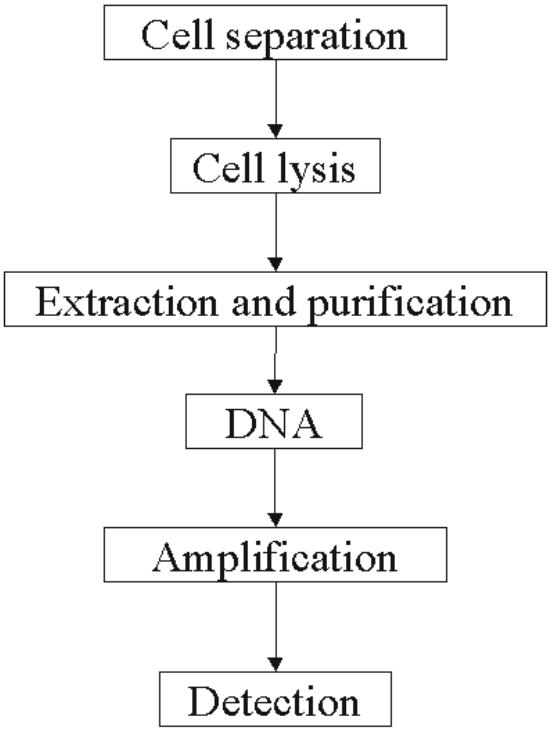

Figure 6. Illustration of conventional sample pretreatment process. Pretreatment is depicted from cell seperation to detection of amplified DNA (Huang, Mather et al. 2002).

1.3 Sample pretreatment

Biological samples like polluted ground water, meat juice, hair, fecal samples or whole blood have a complex composition. The function of the pretreatment is to either dissociate the complex material into it's main components, or to separate the desired starting material from the rest of the raw sample. If a sample contains particles that can block the analysis system, then the sample pretreatment can simply be to remove those particles. The pretreatment process can be illustrated as in Figure 6, where a series of steps ends with a detection signal from the performed analysis. Due to the diverse origin of samples such as forensic investigations, environmental studies or clinical diagnosis, no general pretreatment method exist. The selected pretreatment method has to be compatible with both the sample and the type of analysis conducted. Therefore, the pretreatment often has to be adapted for the specific use (Lichtenberg, de Rooij et al. 2002). This adaptation of analysis, sample, and pretreatment method are in integrated microsystems even extended further, as the entire system has to be designed to apply for the particular combination of pretreatment, sample, and analysis.

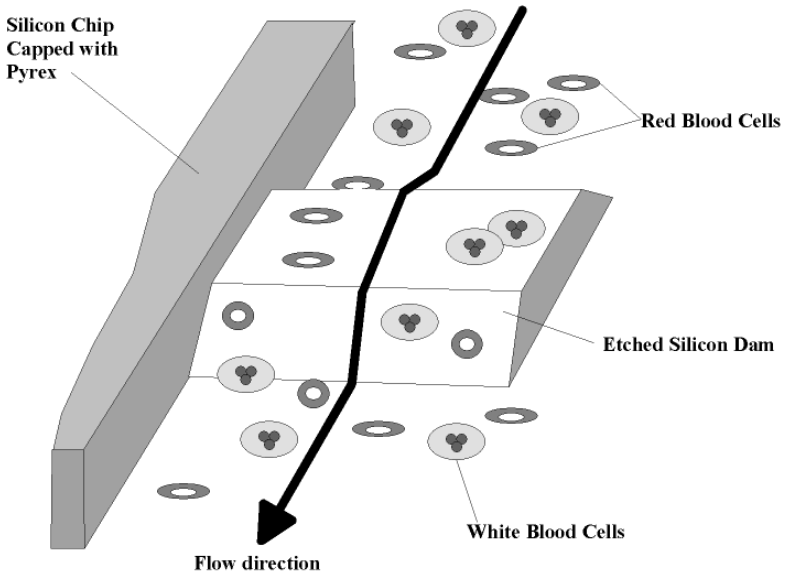

Figure 7. Schematic illustration of 'weir-type' filter. A gap between top of the etched silicon dam and the pyrex glass cover, provides the active filtration of cells based on size. The arrow indicates the flow direction (Wilding, Kricka et al. 1998).

1.3.1 Sample pretreatment on-chip

Sample pretreatment in microsystems is a field that has been pursued with less vigor than the development of on-chip assays, although pretreatment is of overwhelming importance for the end user (Huang, Mather et al. 2002). Several conventional preparation methods exist (Wilson 1997; Huang, Mather et al. 2002; Lichtenberg, de Rooij et al. 2002), but not all of them are suitable for integration in microchips. Below are given two examples of pretreatments to illustrate the challenges that arise when attempting to apply pretreatment on chip.

The first example is filtration. Many conventional filters exist, but the incorporation of these filters into a microchip is not a straightforward process, and requires custom made fittings. Several filter designs have been fabricated in micro scale (Wilding, Kricka et al. 1998), but the practical use of these designs might be limited due to their complicated fabrication process (Lichtenberg, de Rooij et al. 2002). One filter design that has proven useful is the ‘weir-type’ filter (Figure 7) (Wilding, Kricka et al. 1998; Lichtenberg, de Rooij et al. 2002). This type of filter has successfully been used in a study to filter white blood cells from whole blood and afterwards amplify the human dystrophin gene by PCR on a microchip. It is, however, only possible to use filters if it is the large-

sized cell fraction that is wanted, as small cells in solution will go past the obstructions that constitute the filter.

A different separation method that has been applied to micro fabricated systems is dielectrophoresis (DEP). This method does not rely on physical obstacles to retain cells, but acts via electric fields that the cells are exposed to when they pass an array of electrodes (Becker, Wang et al. 1994; Markx, Dyda et al. 1996). The electric fields exert a dielectrophoretic force on the cells, as they pass by. This DEP force is dependent on the dielectrophoretic characteristic of the cells. The dielectrophoretic characteristic is determined by the cellular structure, i.e. cellular organelles and the composition of the cellular membrane (Goater and Pethig 1998). It is therefore possible to selectively capture a specific cell type by DEP. Dielectrophoresis has been used for several separations of biological materials ranging from separation of bacteria species (Markx, Dyda et al. 1996) to separation of cancer cells from other blood cells (Becker, Wang et al. 1994; Gascoyne, Wang et al. 1997). In addition, there are no size requirements for DEP, and if the right settings are applied, molecules can also be held back (Asbury, Diercks et al. 2002). Furthermore, DEP has the advantage of fast response time as the separation force can be completely eliminated simply by turning off the power. The features and possibilities of DEP are superior to other methods (Ohhara, Kurosu et al. 1994; Harmon, Ransom et al. 1997; Wilding, Kricka et al. 1998). Therefore, the possibility to apply DEP as a pretreatment for PCR has been investigated (Chapter 6).

1.3.2 Sample pretreatment for PCR

Pretreatment of samples for PCR is often necessary because DNA has to be accessible for the DNA polymerase, and because several factors exist that can inhibit activity of DNA polymerase. Overcoming inhibition is dependent on the way inhibition happens. Generally there are three reasons for failure of PCR. These are inadequate lysis of cells, degradation or capture of DNA, or inhibition of DNA polymerase. If the inhibition happens because of inadequate lysis of cells, a useful treatment is enzymatic degradation of the embedding tissue (Dieffenbach and Dveksler 1995; Huang, Mather et al. 2002). Break down of template DNA can be another reason for lack of amplification (Wilson 1997). DNA degradation can happen after mechanical lysis of cells if the sample is not treated appropriate, as DNA degrading enzymes are liberated from the intracellular compartment.

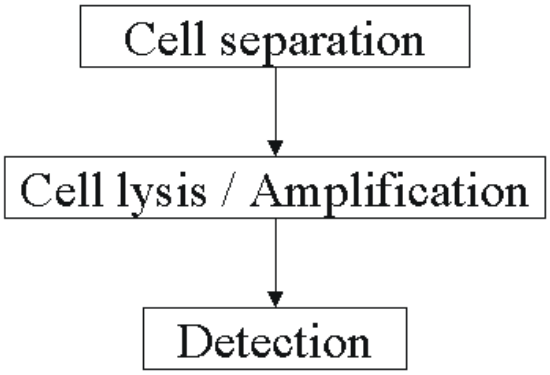

Figure 8. Illustration of a simple sample pretreatment process. Pretreatment is depicted from cell seperation to detection of amplified DNA. The process can be simplified due to elimination of process steps in an integrated microsystem.

Inhibition can also be inflicted directly on DNA polymerase. A common way to overcome this effect is to dilute the sample (Wilson 1997). The essence of this method is to dilute the inhibitor so the concentration is below the inhibition threshold. However, the DNA is also diluted by this treatment. Nevertheless, if just a trace amount of DNA is left in solution, then PCR is still possible due to the inherent powerful amplification of the PCR technique. In addition to dilution, sample pretreatment to overcome inhibition on polymerase also includes DNA extraction and different washing techniques (Huang, Mather et al. 2002). A new microsystem with integrated PCR and sample pretreatment has recently been designed and fabricated in our group (El-Ali, Perch-Nielsen et al. 2003). The new system uses a washing method as the pretreatment for PCR (Perch-Nielsen 2003). With this device some of the steps in the pretreatment are eliminated (Figure 8), and the overall time for analysis can be decreased considerably.

Another, but for the most part neglected, alternative is to choose a more appropriate DNA polymerase for the particular sample and PCR application. In addition to *Taq*, several other DNA polymerases exist. The different DNA polymerases have different thresholds for inhibition from substances like blood, feces and meat (Abu Al-Soud and Radstrom 1998). For example, it is reported that *rTth* DNA polymerase is 100 times more resistant to hemoglobin than *Taq* (Abu al-Soud and Radstrom 2001). Hemoglobin is the oxygen-transporting compound found in erythrocytes (red blood cells). A similar difference in inhibition threshold of polymerases might exist for compounds related to microchip fabrication, and therefore some polymerases might be more suitable for use in microchips than others.

1.4 Outline of thesis

This work presents and discusses two different biological assays applied in microfabricated systems. The first biological application is flow cytometry suited for cellular assays. However, the main emphasis is on polymerase chain reaction (PCR) and coating. Coating is a prerequisite for performing PCR in microstructures.

- In Chapter 2, a micro flow cytometry device is described and discussed. It is used for an acute toxicity assay of cells exposed to the detergent Triton X-100. Mixing is accomplished on-chip that also allows for a dynamic assay.
- Chapter 3 describes investigations of surface coating performed during the development of the PCR chip.
- In Chapter 4 two important circumstances concerning PCR on-chip are presented: Heating and alternative fabrication materials. These concerns are presented in a study of simulation and validation of a SU-8 based PCR thermo cycler chip.
- In Chapter 5 another study illustrates and discusses the utilization of a PCR chip for detection of *Campylobacter*.
- In Chapter 6 an implementation of sample pretreatment on micro scale is described and discussed. DEP is chosen as the pretreatment method, due to the superior features and possibilities of this technique.

2 Flow cytometry

Static and Dynamic Acute Cytotoxicity Assays on Microfluidic Devices[1]

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Abstract

Static and dynamic acute toxicity assays of cells were performed on microfluidic devices. For the static assay on the cross microchip, the cells were incubated with an agent (Triton X-100) in the cell reservoir, and the cells were focused into a narrow stream at the cross intersection and detected 3 mm downstream. Cell viability was determined using the ratio of fluorescence signals from a live cell stain (calcein) and a dead cell stain (propidium iodide), and cells were considered alive if this ratio exceeded unity. The mortality of the cells was determined as a function of the Triton X-100 concentration, and a cumulative LC50 value of 138 μM Triton X-100 was obtained for an incubation period of 7 to 12 min. For the dynamic assay on the spiral microchip, the cells were mixed with Triton X-100 at the cross intersection, allowed to incubate while being transported down the spiral analysis channel, and detected at 1, 120, and 220 mm downstream from the cross intersection. With an average cell velocity of 4.7 mm/s, these detection points corresponded to incubation times of 0.2, 25, and 47 s, and the LC50 values for the 25 and 47 s incubation times were 290 and 250 μM Triton X-100, respectively. Higher LC50 values for the dynamic assay were expected due to the shorter incubation times. The cells and buffers were transported through the channels by applying a subambient pressure to the waste reservoir.

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Introduction

Rapid and accurate single cell assays of large cell populations are important in biology and medicine. Flow cytometry is a common and well established method for counting and sorting particles or cells on a large scale [1], and a variety of assays can be performed using this technique [2-4]. With flow cytometry, particles or cells are hydrodynamically focused and probed individually by an incident light source. Elastic and inelastic light scattering signals are detected and analyzed to provide cellular information and population distributions. Although flow cytometry can provide information at incredible rates (up to 50 kHz), the assays tend to be labor intensive and require skill. Microfluidic devices (microchips) have the potential to enhance the precision and throughput of these single cell assays by integrating and automating cell handling, processing, and analysis steps. Microfluidic devices fabricated on planar substrates are advantageous for manipulating small sample volumes, rapidly processing materials, and integrating sample pretreatment and separation strategies [5, 6]. To carry out a complete assay, different functional elements can be designed and serially integrated on microchips, and in the case of cell assays, include mixers, incubators, cytometers, and sorters coupled together under computer control.

Miniature flow cytometers were first reported in the 1960's [7, 8] and were used to compare absorbance signals from carcinomatous and normal cells from different sites in the human body. More recently, microfluidic devices have been designed and tested for hydrodynamically focusing fluid streams [9] and for the analysis of fluorescently labeled leukocytes [10]. In addition to these hydrodynamic schemes, cells and particles can be manipulated using electric fields. Negative dielectrophoresis has been demonstrated on a microchip to sort and trap latex particles and cells using two- [11] and three-dimensional [12] structures. Electroosmotic transport was used to mix a lysing agent with canine red blood cells on a microfluidic device [13], and electrokinetic focusing was used to spatially confine fluids [14], latex particles [15], and *E. coli* cells [16]. In addition, electrokinetic transport and sorting of fluorescently labeled *E. coli* have been reported [17, 18]. For cell-based reactions on microfluidic devices, cellular membrane potentials were measured by mixing samples with cells and monitoring the membrane potential using potential-sensitive dyes [19]. Also, a microfabricated coaxial mixer was used to rapidly combine cells with reagents, and the output of the mixer was connected to a commercial flow cytometer [20]. Toward increased integration, sample focusing and sorting have been coupled with a miniaturized fluorescence detection system [21], and microfabricated components for coaxial focusing flow,

cell culturing, and detection optics have been evaluated [22]. This work has also shown cell throughputs up to 12 kHz.

Such devices have the potential to significantly reduce the size of flow cytometers and increase cell throughput because many devices can be fabricated on a single substrate and operated in parallel. Carryover and contamination can be eliminated as the microfluidic parts can be fabricated inexpensively and would be disposable. This is especially advantageous for handling hazardous samples. In addition, for rare cell sorting the dilution factor in the collection reservoir on a microfluidic cytometer can be orders of magnitude smaller than for a standard bench-scale flow cytometer. Presently, the cell throughputs for microfluidic cellular assays are lower than for bench-scale cytometers, but substantial progress is being made. However, microfluidic devices offer the possibility of easily integrating precise mixing of cells with reagents and controlled incubation times to cytometric analysis.

To demonstrate some of these features, we report static and dynamic acute cytotoxicity assays performed on microfluidic devices. For the static assay on the cross microchip, the cells were incubated with Triton X-100 in the cell reservoir, focused at the cross intersection, and detected. For the dynamic assay on the spiral microchip, the cells were mixed with Triton X-100 at the cross intersection, incubated in spiral analysis channel, and detected at three locations along the analysis channel. For both assays, cell viability was determined using the ratio of fluorescence signals from a live cell stain and a dead cell stain. Cell mortality was determined as a function of the Triton X-100 concentration, and LC50 values were calculated under both static and dynamic conditions.

Experimental Section

Microchip Fabrication. The microchips depicted in Figure 1 were fabricated using standard micromachining methods. Briefly, the microchip substrates were white crown glass coated with chromium (100 nm), an anti-reflective coating, and a positive photoresist (SL-4006-2C-AR3-AZ1350; Hoya Corp., Tokyo, Japan). The microchip design was transferred from a commercially fabricated photomask (HTA Photomask, San Jose, CA) onto the substrate by UV flood exposure (J200; OAI, Milpitas, CA). The photoresist was developed (MF-319 Developer; Shipley, Marlborough, MA) followed by etching the chromium film (Chromium Etchant; Transene, Danvers, MA). The channels were then etched into the substrate using a dilute, stirred HF/NH_4F solution (Buffered Oxide Etchant; Transene). Prior to bonding the coverplate, 3-mm diameter

access holes were ultrasonically drilled at the ends of the etched channels (Sonic Mill, Albuquerque, NM), and the substrate was diced (Basic Dice II; Dicing Technology, Longwood, FL). The drilled substrate and a crown glass coverplate were then hydrolyzed, joined, and thermally annealed at 550° C for 10 hours to permanently mate the substrate and coverplate. Short segments of glass tubing (6 mm o.d. x 4 mm i.d. x 6 mm length) were epoxied over the drilled holes to serve as fluid reservoirs for the cells, buffer 1, and buffer 2. The waste reservoir did not have a fluid reservoir attached. The cross microchip dimensions were 25 x 50 x 3 mm, and all channels were 16 μm deep. The analysis channel was 43 μm wide at half-depth, and the cell, buffer 1, and buffer 2 channels were 43 μm wide at half-depth and expanded to 220 μm wide at half-depth 500 μm from the cross intersection. The spiral microchip dimensions were 50 x 50 x 3 mm, and all channels were 18 μm deep. The analysis channel was 41 μm wide at half-depth, and the cell, buffer 1, and buffer 2 channels were 41 μm wide at half-depth and expanded to 220 μm wide at half-depth 500 μm from the cross intersection. The channel dimensions were measured prior to bonding the coverplate using a surface profiler (P-10; Tencor, Mountain View, CA).

Channel Coating. To minimize cell adhesion to the channel walls of the microchip, the channels were coated with poly(dimethylacrylamide). The channels were sequentially rinsed with 1 M NaOH for 5 min, water (18 MΩ-cm, Barnstead International, Dubuque, IA) for 5 min, 1% (v/v) γ-methacryloxypropyltrimethoxysilane (Sigma, St. Louis, MO) in water with 0.4% (v/v) acetic acid for 30 min, and water for 2 hr. Next, the channels were filled with 4% (w/v) dimethylacrylamide in 0.5x Tris-Borate-EDTA buffer (Sigma) with 0.06% (w/v) ammonium persulfate (Aldrich) and 0.4% (v/v) N,N,N',N'-tetramethylethylenediamine (Aldrich). The chips were stored overnight at 4°C wrapped in parafilm to reduce evaporation and rinsed the following day with water.

Cells and Buffers. The Jurkat cells, strain ATCC# TIB-152 (American Type Culture Collection, Rockville, MD), used in this study were grown in RPMI 1640 media (GibcoBRL) supplemented with 10% (v/v) fetal calf serum, penicillin (100 IU/mL), streptomycin (100 μg/mL) and L-glutamine (200 μM). The cells were stored in a 5% CO_2 (v/v) atmosphere. For the assays, 2 mL of confluent cell suspension were spun down at 3000 rpm for 3 min, and the cells were resuspended in 994 μL Cell Dissociation Solution (prepared in phosphate buffered saline; Sigma) enriched with D-glucose (1 mg/mL). Calcein AM (1 μL at 1 mg/mL in DMSO; Molecular Probes, Eugene, OR) was added to the cell solution and incubated for 10 min at room temperature.

Propidium iodide (5 μL at 2 mg/mL in DMSO; Molecular Probes) was then added to the cell solution.

For the static assays on the cross microchip, Triton X-100 was added to the cell solution to a final concentration of 0 to 220 μM, and the cell solution was placed in the cell reservoir. Cell Dissociation Solution was put into the buffer 1, buffer 2, and waste reservoirs. For the dynamic assays on the spiral microchip, the cell solution without Triton X-100 was placed in the cell reservoir. Cell Dissociation Solution with 0 to 740 μM was put into the buffer 1 and 2 reservoirs, and Cell Dissociation Solution without Triton X-100 into the waste reservoir.

Microchip Operation. The microchip was inserted into a microchip cartridge made from PEEK to allow a subambient pressure to be applied to the waste reservoir. The chip cartridge contained a waste reservoir that was coupled to the microchips with an o-ring and was threaded to accept Cheminert PEEK fittings (Valco Instruments Co., Houston, TX). Polytetrafluoroethylene (PTFE) tubing (0.250 mm i.d., Valco Instruments Co.) and a 1/4-28 Cheminert PEEK fitting were used to connect the microchip to a syringe pump (55-2226; Harvard Apparatus, Holliston, MA) with a 30 mL syringe. To apply a subambient pressure to the waste reservoir and transport cells and buffers through the channels, the syringe was withdrawn to a differential pressure reading of 0.68 bar below ambient. The flow of cells and buffer at the cross intersection are shown in Figure 2. The pressure was measured using a pressure transducer (PX186-015BD5V; Omega Engineering, Stamford, CT) with a multimeter for readout.

Detection. For measurements at various detection points along the analysis channels, the 488-nm line from an argon ion laser (I-90-5; Coherent, Santa Clara, CA) was focused with a 150 mm focal length lens onto the channel. The fluorescence from the hydrolyzed calcein AM and propidium iodide was collected with a 40x microscope objective (CD-240-M40X; Creative Devices, Neshanic Station, NJ) and projected onto a spatial filter with a 200 μm slit. The fluorescence signal was passed through a 488 nm notch filter (Kaiser Optical Systems, Ann Arbor, MI) and split with a 590 nm dichroic filter (590DRLP-9926; Omega Optical, Brattleboro, VT). The calcein signal reflected by the dichroic filter was passed through a 530 nm band-pass filter (530DF30, Omega Optical), and the propidium iodide signal transmitted by the dichroic filter was passed through a 635 nm band-pass filter (635DF55, Omega Optical). The signals were detected by two photomultiplier tubes (PMTs; R928, Hamamatsu Corporation Bridgewater, NJ), amplified using low noise current preamplifiers (SR570, Stanford Research Systems, Sunnyvale, CA), and read into a computer (G3-300MHz; Apple, Cupertino, CA) using a multifunction I/O card (PCI-

MIO 16-XE50; National Instruments, Austin, TX). For the static assay on the cross microchip, the detection point was 3 mm downstream from the cross intersection, and for the dynamic assay on the spiral microchip, detection points were 1, 120, and 220 mm downstream from the cross intersection. Data for the propidium iodide and calcein detection channels were acquired at 3 kHz.

Optical images were obtained using an inverted microscope (TE300; Nikon, Melville, NY) equipped with a 10x objective, a high pressure mercury lamp, and a CCD camera (NTE/CCD-512-EBFT; Roper Scientific, Trenton, NJ). The CCD images were acquired using IPLab Spectrum (Scanalytics Corp., Vienna, VA). To determine the mixing ratio of the cells with buffers 1 and 2 at the cross intersection of the spiral microchip, images of fluorescein in the cell channel were compared with fluorescein in all of the channels. The flow from the cell channel comprised 36.5% of the flow into the analysis channel, and flow from the buffer 1 and 2 channels made up 63.5% of the flow into the analysis channel.

Data Analysis. Peak detection and analysis were performed using LabVIEW. For each detection channel, a peak find routine was implemented independently, peaks in each channel were then correlated, and the ratio of the calcein peak height to propidium iodide peak height was calculated. For competent (living) cells the ratio of the calcein to propidium iodide signals was >> 1 while for compromised cells the ratio was << 1. Once the cell membrane was compromised, the change in peak height ratio from >> 1 to << 1 was rapid and usually less than 0.5 s [16]. To differentiate between competent and compromised cells, an arbitrary calcein to propidium iodide peak height ratio of 1 was selected. Concentration-response curves generated from the LabVIEW-based analysis were plotted in IGOR Pro (Wavemetrics, Lake Oswego, OR). The data were then fitted using a sigmoidal function of the form:

(1) $$y = y_{baseline} + \frac{y_{\max}}{1 + \exp\left(\frac{(x_{1/2} - x)}{rate}\right)}$$

where $y_{baseline}$ is the minimum value of y, y_{max} is the maximun value of y, $x_{1/2}$ is the value of x at $(y_{max} - y_{baseline})/2$, and rate is the fitting variable which determines the rise rate of the sigmoid.

Results and Discussion

The assays were conducted under static and dynamic conditions. The static assays were performed to establish a baseline for the quality of information that could be obtained from cytotoxicity assays on microfluidic devices. For the static assay, cells were incubated in a buffer with Triton X-100, transported to the cross intersection, and focused in the lateral dimension by flow from buffer

1 and 2 channels. Figure 2 shows the flow of cells at the cross intersection. The cells are focused into a narrow stream approximately 15 μm wide and detected 3 mm downstream of the focusing. Cell viability as a function of Triton X-100 concentration was determined by comparing the fluorescence signals from calcein and propidium iodide. If the ratio of calcein to propidium iodide signal was greater than one, the cell was considered alive, and if the ratio was less than one, the cell was dead. The fluorescence from the calcein and propidium iodide was collected as the cell traversed the detection region. Calcein AM is membrane permeable and diffuses into cells where the acetoxy groups are hydrolyzed by non-specific esterases. The hydrolyzed dye (calcein) is both cell membrane impermeable and fluorescent. If the cell membrane is compromised by the Triton X-100, the calcein diffuses out of the cell reducing the calcein signal. Propidium iodide is a membrane impermeable dye. When the cell membrane is compromised by the Triton X-100, the propidium iodide diffuses into the cell interior and binds with DNA and RNA. Upon binding the fluorescence quantum yield increases substantially. The fluorescence signals were not normalized for relative concentration or quantum efficiency.

Data from the static cell assays are shown in Figure 3, and the signals from the calcein and propidium iodide are offset for clarity. The labels L and D mark cells that were considered live and dead, respectively. Figure 3 shows 10 cells analyzed in a 1 s period with 6 live cells and 4 dead cells. Typical runs were 30 s in duration and analyzed between 50 and 330 cells per run with cell frequencies from 1.5 to 6.9 Hz. Over 28000 cells were analyzed for the static assays. For the static assays, the cell frequencies and throughputs are listed in Table 1 for the five days. The cells were typically incubated with Triton X-100 in the cell reservoir for 7 to 12 min prior to being analyzed. No change in cell mortality was observed over this incubation period.

The results from the static assays are compiled in Figure 4 showing the variation of cell mortality (% dead cells) with Triton X-100 concentration. On each of five days, between 10 and 12 concentrations of Triton X-100 were evaluated. As seen in Figure 4, the baseline mortality rate for the culture was non-zero and ranged from 7 to 13% dead cells. The entire data set was fitted with a sigmoidal curve to determine the LC50 value (lethal concentration for 50% mortality), and the cumulative LC50 value was 138 μM Triton X-100. The cumulative fit is shown in Figure 4. The data from each day were also fitted to determine the LC50 value for that day, and these values are listed in Table 1. The determination of the LC50 values did not include baseline mortality of the culture without exposure. Over the five day period the relative standard deviation for the LC50 values was 5%. Although no LC50 values for Jurkat cells have been reported in the literature,

LC50 values for a similar immortalized cell line are available. The LC50 value for B16 melanoma cells exposed to Triton X-100 was reported to be 130 μM [23] which is consistent with the 138 μM determined by the microchip static assay.

The quality of the data from the static assays led us to consider performing dynamic assays with the spiral channel design and a controlled incubation time. The cells were mixed with Triton X-100 at the cross intersection, allowed to incubate while traveling down the analysis channel, and can be monitored at any location in the analysis channel. The spiral analysis channel was 25 cm long and allowed the cells sufficient time to incubate with the Triton X-100. Similar to Figure 2, the cells were mixed with buffer containing Triton X-100 at the cross intersection. From the comparison of fluorescence images, the cell buffer constituted 36.5% of the flow into the analysis channel, and buffers 1 and 2 with Triton X-100 comprised 63.5% of the flow. With an analysis channel width of 41 μm, the cell buffer stream was approximately 15 μm wide. With buffers 1 and 2 present on both sides of the cell buffer, the Triton X-100 diffused only 7.5 μm to mix with the cells. Using an approximate diffusion coefficient of 10^{-6} cm^2/s, Triton X-100 should diffusively mix with the cell buffer in under 300 ms. Fluorescence images taken 1 mm downstream from the cross intersection confirm that fluorescein in the cell channel mixed with buffer from the buffer 1 and 2 channels. Although slightly larger (576.6 g/mol molecular weight), Triton X-100 was assumed to mix on a similar timescale as fluorescein.

As with the static assays, the ratio of the calcein signal to propidium iodide signal was used to monitor cell viability. To determine the reaction times for the three detection points, the average cell velocity was calculated from the transit time of the cells through the detection region. The axial length of the detection window was 5 μm, the average cell diameter was 8 μm, and the average baseline peak width for the calcein signal was 2.8 ms. From these values, an average velocity of 4.7 mm/s was estimated corresponding to incubation times of 0.2, 25, and 47 s for the 1, 120, and 220 mm detection points, respectively. Over the course of three days, four data sets were taken with eight Triton X-100 concentrations run on each day.

The results of the dynamic assays are summarized in Figure 5, and the variation of cell mortality (% dead cells) with Triton X-100 concentration for detection points at 1, 120, and 220 mm are plotted. As expected the shortest reaction time at the 1 mm detection point did not produce any compromised cells above the baseline cell mortality in the culture (17% dead cells). For the 25 and 47 s reaction times at the 120 and 220 mm detection points, respectively, cell mortality was observed with average LC50 values of 290 and 250 μM. As expected, the LC50 value for the 25 s

incubation time was greater than for the 47 s reaction time. For clarity of presentation in Figure 5, the data at each Triton X-100 concentration were averaged for the four data sets, and the standard deviations are plotted. The standard deviations in the transition regions where the Triton X-100 began to compromise the cell membranes were large due to day to day fluctuations in cell response to the Triton X-100. In addition, the cell mortality did not rise above the baseline cell mortality as the cells were transported down the spiral analysis channel. This can be observed for Triton X-100 concentrations less than 200 μM in Figure 5 where the baseline cell mortality was within experimental error for the 1, 120, and 220 mm detection points. Over the course of the dynamic assays, the incubation times were held constant by assuring that the subambient pressure applied by the syringe pump remained constant. The pressure fluctuation at the waste reservoir was less than 0.41% over the course of the runs. The cell frequencies for the dynamic assays were 1.5 to 2.6 Hz and over 11000 cells were analyzed providing sufficient throughput for statistics. The cell frequencies and throughputs for the four data sets are listed in Table 2.

In summary, static and dynamic cytotoxicity assays were successfully implemented on microfluidic devices. For the static assays on the cross microchip, the data over the five day period exhibited minimal variation, and the cumulative LC50 value compared favorably with results using a conventional toxicity assay where Trypan blue stained cells were counted in a Bürker chamber. For the dynamic assays on the spiral microchip, cells were precisely mixed with the reagent, and the extended analysis channel enabled monitoring of the cells over a range of incubation times. Future investigations for such cell-based assays will include increasing cell throughput and determining reaction kinetics. These assays along with results from other groups demonstrate the potential of microfluidic devices for rapid and accurate cellular assays.

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Table 1. LC50 Values, Cell Frequencies, and Cell Throughputs for the Static Assays on the Cross Microchip

Day	LC50 Value (μM)	Frequency (Hz)	Throughput
1	139	2.7	4790
2	140	1.5	2870
3	142	3.0	5340
4	133	2.5	4820
5	134	6.9	10850

Table 2. Cell Frequencies and Cell Throughputs for the Dynamic Assays on the Spiral Microchip

Day	Frequency (Hz)	Throughput
1	1.6	1900
2	1.5	1650
3a	1.9	3320
3b	2.6	4400

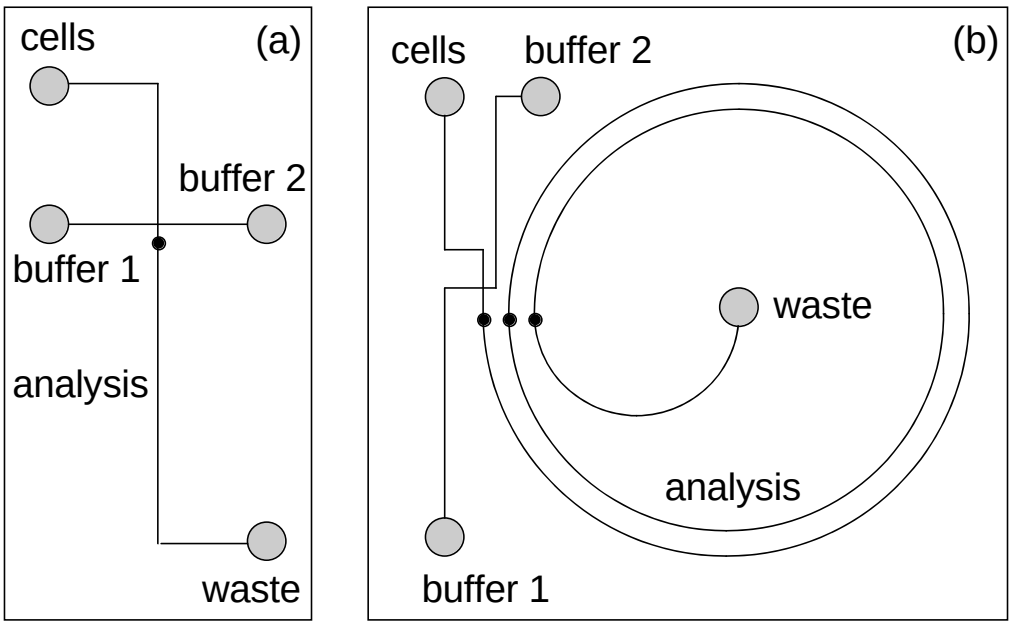

Figure 1. Schematics of (a) cross and (b) spiral microchips used for the acute cytotoxicity assays. The black dots in the analysis channel indicate detection points at 3 mm downstream from the cross intersection on the cross microchip and at 1, 120, and 220 mm downstream from the cross intersection on the spiral microchip. Subambient pressures applied to the waste reservoir transported cells and buffers through the channels.

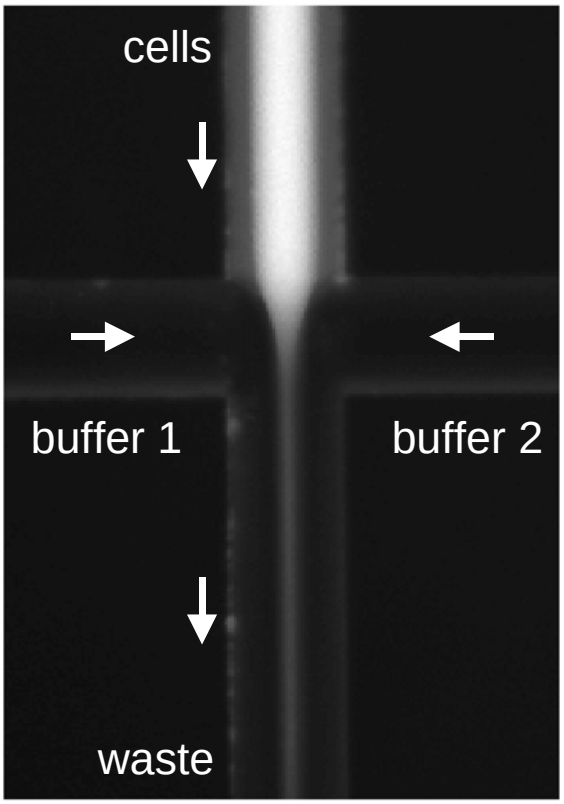

Figure 2. Fluorescence image of labeled cells being transported through the cross intersection. The exposure time was 5 s allowing approximately 100 cells to transit the field of view. The cells were labeled with calcein and transported through the cross intersection by applying a subambient pressure to the waste reservoir. For the static assay on the cross microchip, flow from the buffer 1 and 2 channels focused the sample prior to detection, and for the dynamic assay on the spiral microchip, buffers 1 and 2 contained Triton X-100 and were combined with the cells at the cross intersection to initiate the assay.

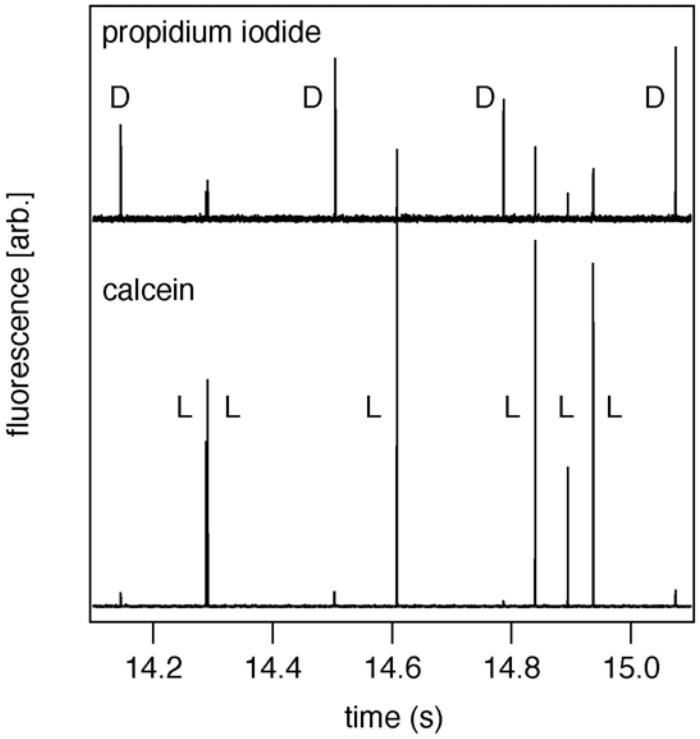

Figure 3. Data acquired using the coincidence fluorescence detection system. The lower trace was from the calcein channel, and the upper trace was from the propidium iodide channel. If the ratio of the calcein to propidium iodide signals was greater than unity, the cell was alive (L), and if less than unity, the cell was dead (D). The data are offset for clarity.

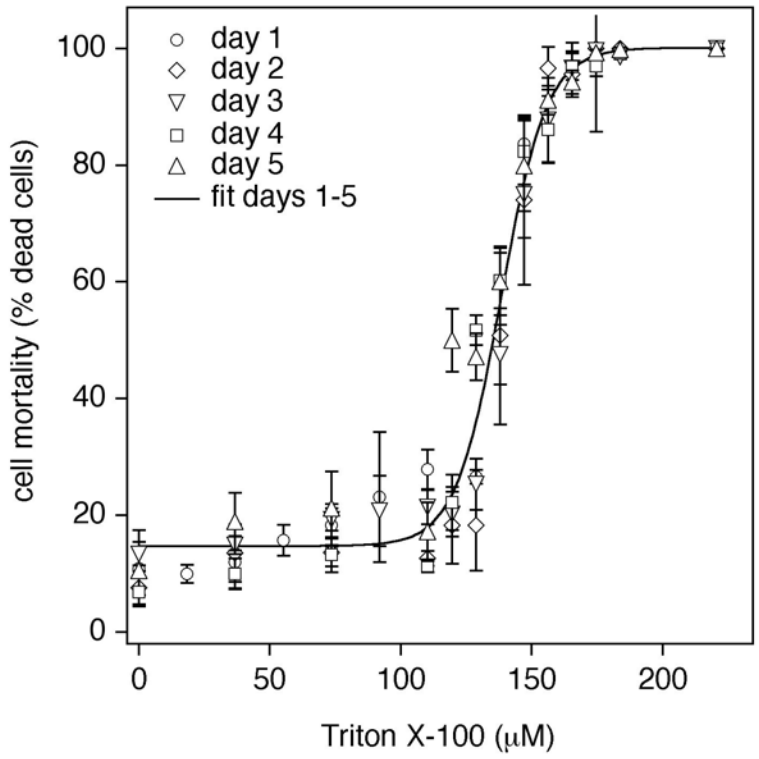

Figure 4. Variation of the cell mortality with Triton X-100 concentration for the static assays performed on the cross microchip. The solid line is the best sigmoidal fit to the data from all five days. See Table 1 for the statistics from days 1 to 5.

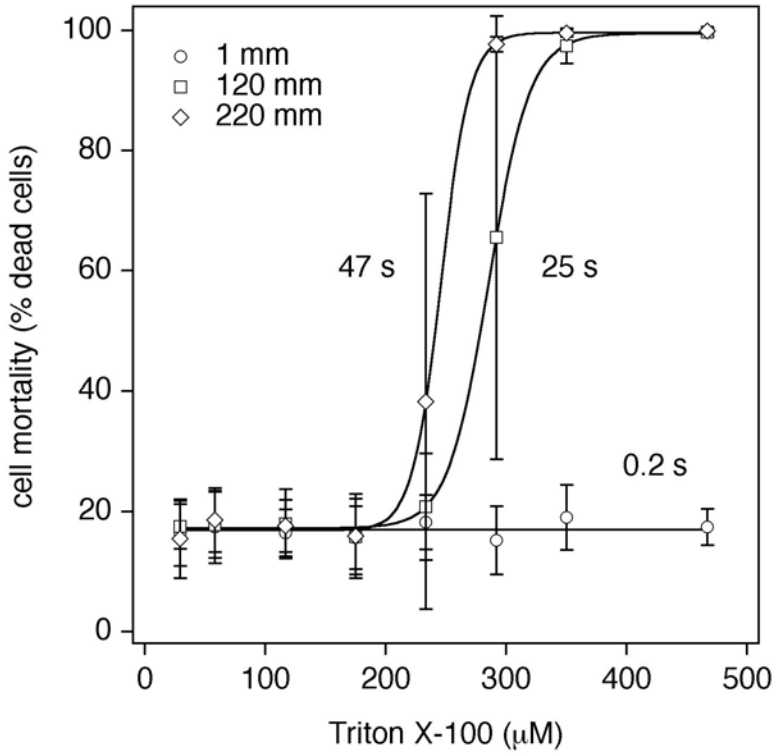

Figure 5. Variation of the cell mortality with Triton X-100 concentration for the dynamic assays performed on the spiral microchip. The data were collected at detection points 1, 120, and 220 mm downstream from the cross intersection. The solid lines are the best sigmoidal fit to data at these detection points corresponding to reactions times of 0.2, 25, and 47 s, respectively. See Table 2 for the statistics for each reaction time.

3 Coating

Miniaturizing analytical devices for μTAS increases the surface to volume ratio. Because of this increased ratio the molecular interaction with the surface become more significant, and surface chemistry will therefore play a dominant role. In the following chapter various surfaces, surface treatments, and coatings are investigated.

3.1 Coating of PCR device

In the early 1990's, surface coatings were investigated for the use of capillary electrophoresis (CE). Later, when PCR was introduced in microstructures, surface properties were again a challenge (Shoffner, Cheng et al. 1996; Giordano, Copeland et al. 2001). Various materials have been used for PCR microreactors. This includes silicon oxide (Cheng, Shoffner et al. 1996; Shoffner, Cheng et al. 1996; Taylor, Winn-Deen et al. 1997; Cheng, Waters et al. 1998; Wilding, Kricka et al. 1998; Lin, Huang et al. 2000), glass (Kopp, de Mello et al. 1998; Oda, Strausbauch et al. 1998; Huhmer and Landers 2000; Khandurina, McKnight et al. 2000; Lagally, Simpson et al. 2000; Schneegass, Brautigam et al. 2001; Sun, Yamaguchi et al. 2002), polyimide (Giordano, Ferrance et al. 2001), and PDMS (Hong, Fujii et al. 2001). However, only in a couple of cases is the PCR compatibility of different surfaces and treatments studied in-depth (Shoffner, Cheng et al. 1996; Giordano, Copeland et al. 2001). Shoffner and colleagues (1996) examined surface passivations by silanization followed by dynamic coating with selected proteins or polymers, and by deposition of nitride or oxide layers onto the silicon surface. Native silicon and silicon nitride (Si_3N_4) were PCR inhibitors. Passivating the PCR chip using a silanizing agent followed by a polymer treatment resulted in good amplification. However, amplification yields were inconsistent and were not always comparable with PCR in conventional tubes. An oxidized silicon (SiO_2) surface gave consistent amplifications with DNA yields comparable to reactions performed in conventional PCR tubes. This surface was, therefore, preferred for the reaction chambers. Giordano and colleagues (2001) examined silanization and other coatings on a glass surface. Polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), and hydroxyethylcellulose (HEC) were used for dynamic coatings, epoxy (poly)dimethylacrylamide (EPDMA) was evaluated as an adsorbed coating, and chlorodimethyloctylsilane was used for silanization. They found surface treatment with PVP, EPDMA, and silanization gave PCR yields in glass tubes comparable to conventional polypropylene PCR tubes, whereas PEG treatment of glass tubes gave a very low

yield. HEC and untreated glass severely inhibited the PCR reaction. Because the silanization technique was time-consuming, coating with EPDMA was preferred.

In summary, studies of surface passivation have independently shown that treatment of the surface in microreactors is necessary in order to have successful amplifications. This is due to the inhibiting property of native silicon, silicon nitride, and glass surfaces. Circumvention of this inhibition is achieved by thermal grown silicon dioxide or by including a dynamic coating compound in the PCR solution.

3.2 Coating of microchips with SU-8 surface

This section describes the actions taken to develop a robust surface coating on microfabricated PCR chips used in this study. The robust surface coating is achieved by silanization of the chip surfaces.

A novel polymer based microreactor with embedded heaters was tested for PCR compatibility (El-Ali 2003). The chip has integrated heaters covered with a thin SU-8 layer, SU-8 walls, and a PDMS lid (Figure 9). Correct temperature control and efficient heating and cooling was demonstrated in this design (El-Ali, Perch-Nielsen et al. 2003). The motivation for the coating experiments were unsuccessful preliminary amplifications on PCR chips. This lack of amplification indicates an interaction with chip surfaces, as some compounds of the PCR solution is either bound to the surface or present in too low an amount for PCR to happen due to surface interactions.

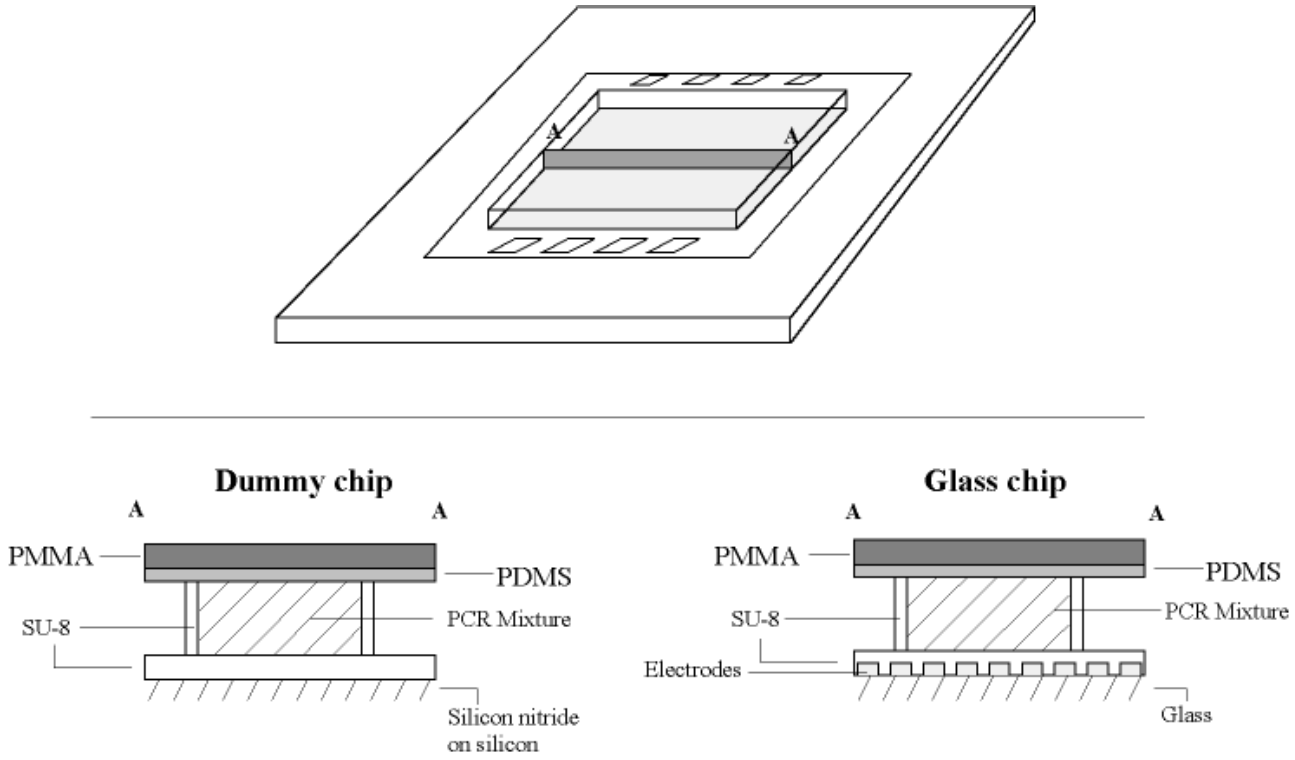

Figure 9. Schematic cross section illustration of PCR chip. The top panel constitute an overview of the PCR chip, and the bottom panel constitute the detailed cross section of Dummy chip with SU-8 on silicon nitride, and Glass chip with SU-8 on glass. The cross section area is depicted in the top panel in a grey shade between the two A's.

3.2.1 Materials and Methods

Bacterial cells and DNA isolation

The DNA used throughout this chapter as template DNA for PCR is isolated from the *Campylobacter jejuni* reference strain CCUG 11284. Bacterial culture conditions are described elsewhere (Bang, Scheutz et al. 2001). In short, a 24 hour CCUG 11284 culture on a blood agar plate (BA; Blood Agar Base No. 2, Oxoid, supplemented with 5% sterile defibrinated calf blood) was grown in micro aerobic conditions (6% O_2, 6% CO_2, 4% H_2, and 84% N_2) at 42°C. Chromosomal DNA was isolated from *Campylobacter* cells from the BA plate using a phenol extraction method described previously (Bang, Pedersen et al. 2001). DNA was eluted in 100 µl sterile-filtered water pre-heated to 65°C. The concentration of *Campylobacter jejuni* chromosomal DNA is 535 µg/ml in the final DNA isolation. The DNA preparations were stored at –20°C until use. The culturing and DNA isolation was performed at Danish Veterinary Laboratory (Aarhus, Denmark).

PCR solution and conditions

Both control experiments performed in conventional thin walled PCR tubes, and experiments performed in PCR chips are prepared in volumes of 25 µl per reaction. The standard PCR solution used in this chapter consists of 1x Taq DNA polymerase buffer (Boehringer-Mannheim, Germany), 0.01 U/µl Taq DNA polymerase (Boehringer-Mannheim), 0.625 mM $MgCl_2$, 0.25 mM of each dNTP, 200 fM of each primer (DNA Technology, Aarhus, Denmark), and 0.5 µg *Campylobacter jejuni* chromosomal DNA. The two used *cad*F gene primers F2B and R1B are previously described in details (Konkel, Gray et al. 1999; Bang, Nielsen et al. 2003). The primer pair F2B and R1B amplifies a 400 bp amplicon. Control experiments performed in tubes were executed in the thermo cycler PTC-200 (MJ Research, Inc., MA, US). The PCR conditions used in this study were similar to those previously described (Konkel, Gray et al. 1999; Bang, Nielsen et al. 2003), i.e. 94°C for 5 minute followed by 40 cycles of 94°C for 1 minute, 45°C for 1 minute and 72°C for 3 minutes. The final step was 72°C for 10 minutes.

Dynamic coating

In order to improve the DNA yield of PCR on-chip, dynamic coating has been addressed in section 3.2.5. Inclusion of 0.25% (w/v) bovine serum albumin (BSA; Sigma, St. Louis, Mo, US) and 0.01 mg/ml salmon sperm DNA (Sigma) in the PCR solution was tested as dynamic coating agents.

DNA analysis

All PCR products were analyzed on the microchip based Agilent 2100 Bioanalyzer (Agilent Technologies, CA, US) using the DNA 500 kit. The DNA 500 kit can analyze DNA fragments up to a length of 500 base pairs. This kit is suited for the amplified *cad*F amplicon of 400 bp. The sizing accuracy of Agilent 2100 Bioanalyzer is specified to be ±10%. Agilent 2100 Bioanalyzer also offers quantification of analyzed samples. The specification states a quantitation accuracy of ±30%.

Chip fabrication

Two PCR designs have been used in this chapter. A glass PCR chip where the reaction chamber is fabricated on glass substrate, and a dummy PCR chip where the reaction chamber is fabricated on silicon substrate (Figure 9). The PCR chip fabrication has been described in details elsewhere (El-Ali 2003). Briefly, in the case of glass PCR chip, the SU-8 PCR chamber was manufactured on a 1mm

thick glass substrate with thin film platinum heaters and temperature sensor integrated on chip for thermo cycling. The chamber dimensions on the PCR chip were 7mm x 7mm x 0.4mm resulting in a chamber volume of 20μL. Thermometer and heater electrodes were patterned by lift-off in a 2000Å platinum layer on the glass substrate. The electrodes were protected from PCR mixture by a 5 μm SU8 layer (XP2005, MicroChem, MA, US) patterned by photolithography. The PCR chamber walls were defined in a 400μm SU-8 layer (XP2075, MicroChem, MA, US). Spinning a 200μm SU-8 layer twice and soft-bake after each spin accomplished the 400μm layer. The glass wafer was finally diced into PCR chips. The lid on the PCR chip is made of a layer of 0.3 mm PDMS (Sylgard 184; Dow Corning, Michigan, US) on a PMMA support (RIAS A/S, Roskilde, Denmark).

The dummy PCR chip was fabricated in a similar way. However, this design does not have integrated heaters or temperature sensors, and are therefore faster to manufacture. In short, a 525μm silicon wafer with a silicon nitride membrane is coated with a 5 μm SU-8 layer. The chamber walls are defined by a 400 μm SU-8 layer as described for the glass PCR chip. The lid is similar to the one used for glass chip.

Chip coating

To avoid binding of PCR materials to the chip surface all chips were coated by a method previously described (Sambrook, Fritsch et al. 1989) with modifications: Dichlorodimethylsilane (Merck-Schuchardt, Hohenbrunn, Germany) was vapor deposited at partial vacuum in a desiccator for one hour. Subsequently the chips were washed twice in ultra pure sterile filtered water for 15 minutes and finally once for 20 minutes. The chips were dried with compressed nitrogen and stored in a dry place at room temperature until use.

3.2.2 Chip content with additional DNA and *Taq* polymerase

Initial examinations of the PCR chip showed no amplification due to surface interactions. Examinations were performed to test whether it was template DNA or *Taq* polymerase that might absorp to the chip surface. Chip contents thermo-cycled 30 times at conditions described in 'PCR solutions and conditions' in section 3.2.1, were split into two normal PCR tubes. To one tube 0.01U *Taq* was added, and to the other tube 0.5μg DNA was added. After another 30 cycles at the same PCR conditions, the contents of the tubes were analyzed. Figure 10 shows both of these amplifications were negative. The absent amplification signals indicate that both *Taq* and DNA

interact with the chip surface and therefore are absent from the PCR solution. However, for some tubes with additional *Taq* polymerase a formation of primer-dimer is observed. The primer-dimer formation is detected as a small 42 bp peak shortly after the 15 bp lower internal marker in the electropherogram of the analysis (Figure 10). This finding indicates that primers are still present in solution. Therefore, it appears that failure of amplification hitherto is caused by lack of *Taq* polymerase and template DNA, probably due to absorption to the chip surface.

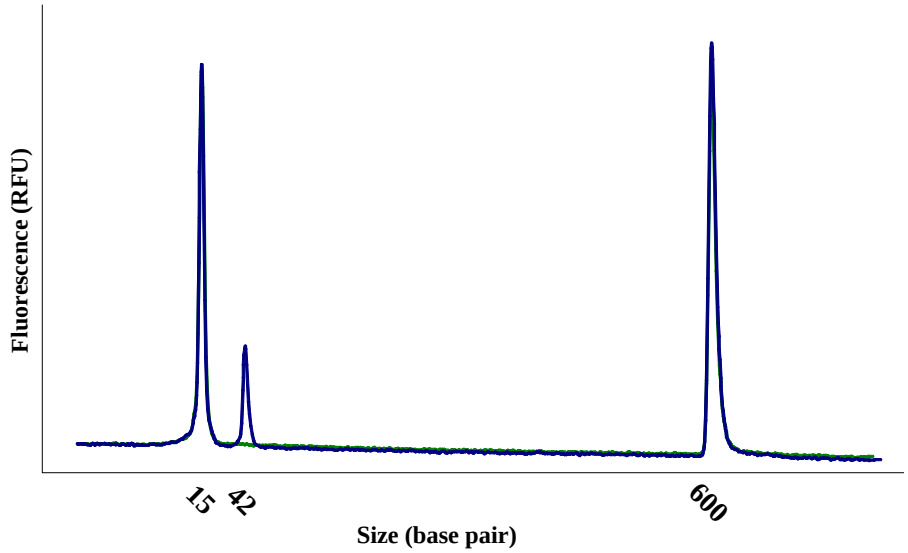

Figure 10. Electropherogram of samples with additional *Taq* and template DNA. The 15 and 600 bp peaks are internal markers from Agilent 2100 Bioanalyzer. The DNA size is mentioned in base pairs (bp), and the fluorescence intensity is measured in relative fluorescence units (RFU). The blue line indicates sample with additional *Taq* polymerase, and green line indicates sample with additional template DNA.

3.2.3 5 times extra primer, DNA, and *Taq* polymerase

In order to overcome unspecific binding on the chip surface observed previously (section 3.2.2), the concentration of DNA template, *Taq* polymerase, or primers were increased five times compared to standard PCR solution (section 3.2.1). 30 cycle amplifications were performed with this solution using external heating by a PTC-200 thermal cycler with an flat bed alpha block (MJ Research, Inc.). Instead of a glass-based chip, these experiments are performed on the silicon-based dummy chip. This alteration is made to ensure correct temperature and an uniform thermal distribution, as silicon has a better thermal conductivity than glass. Simulations have confirmed that this is obtained when using silicon-based dummy chips (Figure 11). Figure 12 shows that amplification is successful if extra DNA and *Taq* is included in the PCR mixture, but not successful if only the primer concentration is raised five times. The insert in Figure 12 indicates

that primers are not bound to the surface as they are detected as a small 20 bp peak immediately after the 15 bp internal marker. These results is in agreement with previous studies, where *Taq* polymerase concentrations in the range of 0.025 to 0.25 U/µl have been reported (Cheng, Waters et al. 1998; Kopp, de Mello et al. 1998; Lagally, Medintz et al. 2001; Schneegass, Brautigam et al. 2001). Five time the standard *Taq* polymerase concentration used in this work is 0.05U/µl, which is in the lower part of the reported range. In summary, these results suggest a combination of extra template DNA and *Taq* polymerase is needed to overcome unspecific binding to the chip surface.

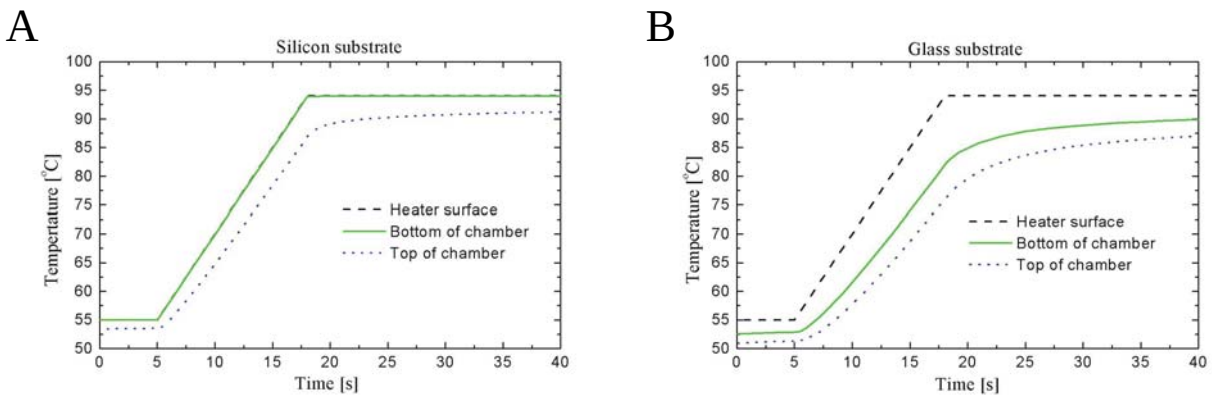

Figure 11. Illustration of dynamic heating simulations in PCR chip designs with silicon substrate (A), and with glass substrate (B). The dashed line indicates the temperature at the heater surface, the green line indicates the temperature on the bottom of the PCR reaction chamber, and the dotted line indicates the temperature at the top of the reaction chamber. Heating is started at time t=5 s in both A and B. The heating rate is set to 3 °C/s, which is according to the specifications of PTC-200 thermal cycler (MJ Research, Inc.) with a 'flat bed' alpha block. The temperature is ramped from 55 °C to 94°C.

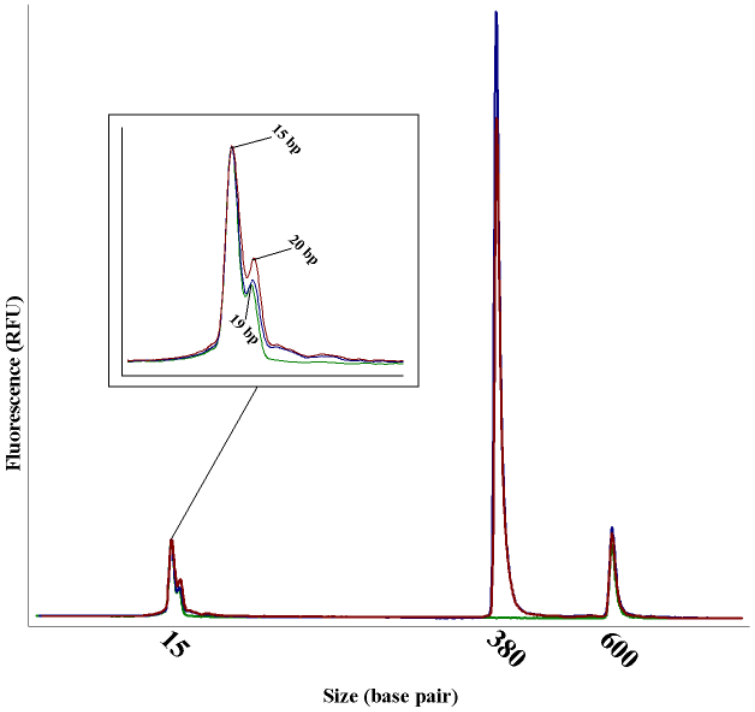

Figure 12. Electropherogram of samples with extra primer, DNA, and *Taq*. The 15 and 600 bp peaks are internal markers from Agilent 2100 Bioanalyzer. The green line indicates sample with five times extra primer added to PCR solution. The blue and red lines both indicate samples with five times extra primer, template DNA, and *Taq* polymerase added to PCR solution. The insert is an enlargement of the 15 bp internal marker and subsequent 19, 20 bp peaks.

3.2.4 Effect of silanization

The need for extra template DNA reduces sensitivity of the performed PCR. Besides, the amount of template DNA may be limited like in the field of forensic science. To reduce the use of template DNA and *Taq* polymerase, an experiment series was conducted with silanization of materials typically used in PCR chips (Northrup, Ching et al. 1993; Lin, Yang et al. 2000; Schabmueller, Lee et al. 2000; Lagally, Emrich et al. 2001; Fuchs, Jeanson et al. 2002). The silanization method is often used in molecular biology (Sambrook, Fritsch et al. 1989). It is easy to use and compatible with all materials tested in the experiments. The silanization experiments are performed on six different substrates: SU-8, PDMS, silicon dioxide (SiO_2), native silicon, silicon nitride (Si_3N_4), and Pyrex glass. Two 2x5 mm pieces of the tested materials were included in the plastic tube with PCR solution. This addition resulted in a surface to volume ratio (SVR) of 3.1 $mm^2/\mu l$. In comparison, the SVR for standard PCR tubes is about 1.5 $mm^2/\mu l$ (Shoffner, Cheng et al. 1996), and 5.6 $mm^2/\mu l$ in the PCR chip demonstrated here (Chapter 4). In contrast to the previous section, standard *Taq* polymerase concentration (0.01 U/μl) was used in the silanization experiments.

Figure 13 shows DNA yields in the presence of the six different materials. The best treatment is silanization with subsequent wash for most of the tested materials. However, PDMS was only compatible with PCR if it was untreated. This is presumably because PDMS takes up the silane compound and liberates it again during PCR. In conclusion, the most effective surface treatment was silanization and subsequent wash, except for PDMS that should be used untreated. If handled this way, all investigated surfaces proved to be compatible with PCR. Furthermore, no extra DNA or *Taq* polymerase had to be added in order to get a successful amplification.

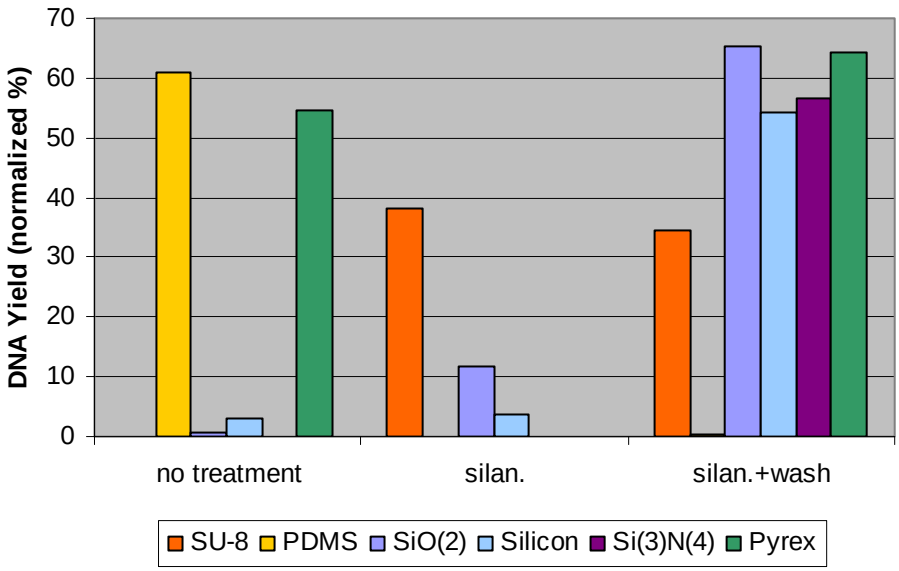

Figure 13. The effect of silanization and wash of various material surfaces typically used in PCR chip. The DNA yields of PCR amplifications are normalized to controls. 'silan.' denotes surfaces that are silanized, and 'silan.+wash' denotes surfaces that are silanized and subsequently washed.

3.2.5 Dynamic coating with BSA and salmon DNA

Enhancers like gelatin, BSA, Tween 20, glycerol, PEG, and PVP are often included in the PCR solution (Taylor, Winn-Deen et al. 1997; Cheng, Waters et al. 1998; Khandurina, McKnight et al. 2000; Yang, Liu et al. 2002). Such substances are added to improve DNA yield.

In the following experiment series it is tested whether or not including BSA and salmon sperm DNA in the PCR mixture will have any effect on the PCR yield, when applied to silicon-based, silanized dummy chips. The PCR in dummy chips are executed with external heating in a PTC-200 thermal cycler with a flat bed block (MJ Research, Inc.). The protein bovine serum albumin (BSA)

is added to overcome unspecific binding of protein (e.g. *Taq* polymerase), and similarly can salmon DNA decrease unspecific binding of DNA (McPherson and Møller 2001). For the PCR solution is either used a undiluted chromosomal DNA isolation (1:1) or and 1:100 dilution of the chromosomal DNA isolation. The PCR yields from the tested microchips were normalized to the positive controls performed in conventional PCR tubes. The results (Table 1) show a positive effect of BSA and salmon DNA if PCR is performed with only 30 cycles. However, at 40 cycles the dynamic coating with BSA and salmon DNA has a negative effect. So, it seems that the initially observed enhancing effect of BSA and salmon DNA was reversed in the last 10 cycles cycles. BSA and salmon DNA was therefore not used further in this study.

Table 1. Yields from PCR microchips with or without dynamic coating. Experiments are performed with different DNA concentrations, at different number of cycles and with or without addition of 0,25 % (w/v) BSA and 0,01 mg/ml salmon sperm DNA. The yields are normalized to control experiments in tubes and shown in percent. At the bottom of each experiment series, the average is shown.

Relative concentration of DNA	**1:100**		**1:1**	
No. of cycles	*30*	*40*	*30*	*40*
- BSA and salmon DNA (%)	34 7	38 33 79	7 14 35 30	82 26 22
	20,50 ± 19,09	50,0 ± 25,24	21,50 ± 13,18	43,33 ± 33,55
+BSA and salmon DNA (%)	47 51 55 54	19 36	14 16 17 59	4 4 6 10 17 3 4 33
	51,75 ± 3,59	27,50 ± 12,02	26,50 ± 21,70	10,13 ± 10,36

3.2.6 PCR on chip

It has been established that inclusion of extra DNA and *Taq* was necessary to overcome the effect of binding to the chip surface. However, silanization could prevent absorption of DNA and *Taq* on pieces of different materials. The final step was to implement the obtained results to achieve successful amplification in the PCR chip with embedded heaters. It was quickly realized that the

Taq concentration could not be reduced. But then again, the *Taq* concentration is for use in standard PCR tubes (Bang, Nielsen et al. 2003), and not in microfabricated chips where the surface properties are not optimized for PCR. In many PCR microchips the volume is several times lower than in tubes (Waters, Jacobson et al. 1998; Khandurina, McKnight et al. 2000; Giordano, Ferrance et al. 2001; Liu, Enzelberger et al. 2002), and the *Taq* polymerase consumption is consequently not high even with 5 times the normal concentration.

In Table 2 are listed the PCR yields obtained from amplifications performed on-chip. Some of the yields are in the range of 8-18% of positive controls. However, these results are from the early experiments where the silanization procedure was not optimized. Later on, more satisfactory and consistent results were obtained. Disregarding the early results, the average of the PCR yields is 68% of the positive controls, but PCR yields of up to 131% have been acquired.

In summary, silanization was necessary in order to prevent unspecific binding of *Taq* DNA polymerase and template DNA to the SU-8 chip surface. Silanization of the PCR chip and addition of extra *Taq* polymerase gave stable, successful amplifications in the PCR chip. All in all, this chip is indeed competitive with other microfabricated PCR chips published so far. The microchip presented here exhibits all the superior features of a PCR microchip, and is competitive in all features.

Table 2. Overview of DNA yields from PCR performed on chips with integrated heaters and thermometer sensor.

Chip number	No. of cycles	Yield on chip (nM)	Yield in tube (nM)	Ratio chip/tube (%)
63	30	11,46	71,92	15,93
64	30	13,06	71,92	18,16
60	40	8,88	107,8	8,24
65	40	55,71	42,48	131,14
66	40	41,47	42,48	97,62
67	40	13,19	27,26	48,39
68	40	14,76	27,26	54,16
70	40	11,34	30,95	36,64
74	40	21,84	22,15	98,60
91	40	41,45	72,22	57,39
95	40	19,01	30,36	62,62
96	40	33,54	80,41	41,71
97	40	14,91	27,45	54,32
98	40	61,97	90,40	68,55

4 Simulation and validation of a SU-8 based PCR thermo cycler chip (PCR I)

Simulation and experimental validation of a SU-8 based PCR thermocycler chip with integrated heaters and temperature sensor[1]

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Keywords: PCR chip, FEM simulation, SU-8

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Abstract

We present a SU-8 based PCR chip with integrated platinum thin film heaters and temperature sensor. The device is fabricated on a glass substrate and the use of SU-8 provides a simple micro fabrication process for the PCR chamber, controllable surface properties and can allow on chip integration to other SU-8 based functional elements. Finite Element Modeling (FEM) and experiments show that the temperature distribution in the PCR chamber is homogeneous and that the chip is capable of fast thermal cycling. With heating and cooling rates of up to 50°C/s and 30°C/s, respectively the performance of the chip is comparable with the best silicon micromachined PCR chips presented. The SU-8 chamber surface was found to be PCR compatible by amplification of yeast gene ribosomal protein S3 and *Campylobacter* gene *cad*F. The PCR compatibility of the chamber surfaces is enhanced by silanization.

[1] Submitted to Sensors and Actuators in September 2002.

Introduction

The Polymerase Chain Reaction (PCR) is an enzymatic method to amplify DNA by repeating a series of thermally controlled reaction steps [1,2]. In the PCR reaction the number of amplified DNA molecules is doubled during each cycle. The thermocyle consist of melting of the double stranded DNA (denaturation) to separate the complementary strands, binding of the specific primers to the target site (annealing), and extension of the primers by a thermostable enzyme such as *Taq* Polymerase. Typical temperatures for the denaturation, annealing and extension steps are 94ºC, 40-72ºC and 72ºC, respectively. Since PCR was first reported in 1986 [1] it has become on of the most widely used techniques in molecular biology, forensic analysis, evolutionary biology, and medical diagnostics. Several PCR chips have been microfabricated of which many rely on relatively complex silicon bulk micromachining for chamber definition [3-6]. One of the advantages of micromachined PCR chips is that the small mass and volume of the systems allows for fast heating and cooling rates of up to 15-80^{o}C/s. The typical power consumption of these devices at 94^{o}C (denaturation temperature) is below 2W. Only few research groups have achieved a high level of integration in their PCR chips. In most groups the integration is limited to heaters and temperature sensors. On chip integration of PCR with other components for either sample pretreatment or post PCR analysis requires process compatibility in the micro fabrication process, which often further complicates the fabrication procedure and adds limitations to the integrated functionality. A few groups have integrated fluidic systems for cell analysis prior to PCR [7] or electrophoretic analysis of the PCR product [8-10] while using external elements for the PCR thermo cycling. However, recently a monolithically integrated PCR and Capillary Electrophoresis (CE) device fabricated on glass chips, with micro fabricated heaters and temperature sensors within the PCR chamber, has been presented [11]. An additional complication in the fabrication of PCR chips is the PCR inhibiting properties of the silicon and silicon nitride surfaces in silicon micromachined reaction chambers [12]. Formations of SiO_2 layer or chemical silanization of the chamber surface have been applied to increase PCR compatibility in such silicon-based devices.

Polymer based microfabricated devices are increasingly being developed as an alternative to silicon and glass based devices. The main drivers of polymer based systems are the good material compatibility with chemical and biochemical assays and the cost advantage that the relatively inexpensive polymer materials can offer compared to silicon and glass based solutions. Polymer based PCR chips using PDMS [13] and polyimide [14] have been presented, both using external

heating elements for the thermo cycling. SU-8 is one of the polymers that is increasingly used in microsystems. SU-8 offers simple fabrication of fluidic components like flow channels, reaction chambers and separation channels. SU-8 is fairly chemically resistant and the surface properties of SU-8 can be easily controlled or altered by plasma or chemical treatment. This makes SU-8 applicable in a large variety of devices. Recently SU-8 has been used in CE devices [15,16] and in DiElectroPhoretic (DEP) devices [17] fabricated on glass substrates. The relative simple fabrication process for most of these SU-8 based systems compared to silicon micromachined ones could help ease the on chip integration with other devices.

In this paper we present a simple way of producing a SU-8 based PCR chip fabricated on a glass substrate. The device consists of a 20µL SU-8 PCR chamber with integrated platinum heaters and temperature sensor for PCR thermo cycling. To our knowledge this is the first example of a PCR chip with a polymer based chamber and integrated heaters and temperature sensor. Chip performance is characterized experimentally and by FEM simulation, using the commercial software packages ANSYS (ANSYS inc) and CFD-ACE (CFD Research Corporation). PCR biocompatibility is tested by amplification of yeast ribosomal protein S3 and *Campylobacter jejuni cad*F genes.

Design and Fabrication

The SU-8 PCR chamber is manufactured on a 1mm thick glass substrate with thin film platinum heaters and temperature sensor for PCR thermocycling integrated on chip, as shown in figure 1. The dimensions of the chamber are 7mm x 7mm x 0.4mm, giving a chamber volume of ~20µL. The heaters are 20 µm wide and 8.2 mm long and are placed with a spacing of 100µm, yielding a total number of 82 heaters in the PCR chamber. The heaters continue beneath and beyond the SU-8 chamber walls to minimize cold wall effects. The heater size and spacing were optimized by FEM simulation, so that temperature distribution in the PCR chamber would be homogeneous. The platinum electrodes are protected from the PCR buffer by a thin SU-8 layer. For sealing of the chip a 500 µm thick glass lid was used. The lid is bonded to the chip using thin coatings of SU-8. A SU-8 coated lid yields a PCR chamber with SU-8 on all sides. The chip is passively cooled by heat conduction through the substrate to a heatsink and by natural convection from the lid.

Fabrication of the chip uses standard MEMS techniques. First an approximately 2000Å thick platinum thermometer and heater electrodes are patterned by lift-off on a 1mm thick glass substrate (Schott). The platinum is deposited using e-beam evaporation with a 100Å titanium layer used as adhesion layer between the platinum and the glass substrate. For electrode protection a 5μm SU-8 (XP2005, Microchem) layer is spun onto the wafer, soft-baked and then patterned using photolithography. The PCR chamber is defined in a 400μm SU-8 (XP2075, Microchem) layer. This layer is applied to the wafer using two spins of 200μm, with a soft-bake step after each spin. After the development of the SU-8 structures in PGMEA (Propylene Glycol Monomethyl Ether Acetate) the substrate is diced into chips. The glass lid is bonded to the chip using SU-8 to SU-8 bonding. SU-8 to SU-8 bonding is done by applying a thin layer of SU-8 to the lid and then partially soft bake this layer. The lid is then put in position on the chip and a final soft bake is done. The bonding is then completed by UV exposure and a post exposure bake of the thin SU-8 layer. The final chip is placed in a ceramic chip carrier for electrical connection through wire bonding. The chip carrier also provides thermal contact between chip and heatsink.

Results and Discussion

To assess the thermal properties of the chip such as temperature homogeneity, power consumption, heating and cooling rates, the performance of the chip has been modeled using a simple analytical 1D heat transport model as well as Finite Element Modeling (FEM) in ANSYS and CFD-ACE. For experimental validation the chip was tested in a setup using a ~15W custom-built power supply with a LabView based PID controller and data acquisition system for thermal cycling.

Figure 2 shows the basic principle of the 1D-model with the material properties used in this model as well as in FEM simulations. The 1D heat transport model assumes that the area spanned by the heaters is at the target temperature 94°C. Heat is transported through the substrate to a heatsink or through the chamber and lid, with natural convection from the lid to the ambient. The model predicts a steady state power consumption of 5.3W and a temperature T_A at the top of the chamber within 1°C of the substrate temperature, thus predicting very uniform temperature distribution in the PCR chamber. 2D and 3D FEM models were used to include edge effects, the effect of discretely placed heaters and to model the dynamic performance of the PCR chip. The 3D CFD-ACE simulation in figure 3 shows that with 100μm heater spacing the spatial temperature distribution in the chamber is very homogeneous. The majority of the chamber is within ±2°C of

the simulated target temperature of 94°C. There is however, a small drop in the temperature at the edge of the chamber. 2D and 3D ANSYS simulations of the temperature distribution in the chamber correspond well to the CFD-ACE simulation. 2D ANSYS simulations predict power consumption at 94°C of 5.5W. This is slightly higher than the simple 1D model. The main reason for this increase is the inclusion of extra heat loss from the edge of the chamber, which is not included in the simple 1D model. The 3D ANSYS and the 3D CFD-ACE FEM simulations both predict a steady state power consumption of 5.7W at 94°C. Again there is a small increase in simulated power consumption when increasing the number of dimensions in the model. The measured steady state power consumption of the chip was ~6.3W at 94°C. This is 10% higher than the 5.7W predicted by FEM simulations. A possible explanation for this is that some fraction of the power is lost in interconnects leading to the heaters.

Although the chip is passively cooled, the 2D ANSYS and the 3D CFD-ACE FEM simulations predict high cooling rates. The ANSYS simulations give a maximum cooling rate of ~35°C/s between 94°C and 80°C and an average cooling rate of ~20°C/s between 94°C and 60°C at the position of the temperature sensor on the substrate. The similar figures from CDF-ACE simulations are ~40°C/s for the maximum cooling rate and ~20°C/s for the average cooling rate. At the top of the chamber, where temperature transition is expected to be slowest, ANSYS simulations predict an average cooling rate of ~10°C/s, or about half the cooling rate on the substrate. If the chip is heated with a power of 11.4W, twice the steady state power needed at 94°C, ANSYS predicts a heating rate from 60°C to 90°C of ~40°C/s at the substrate and ~15°C/s at the top of the chamber. The simulations thus show that the chip can achieve very fast temperature transitions. In figure 4 a measurement of the temperature drop when the power to the chip is turned off is shown. The temperature drop predicted by 3D CFD-ACE and 2D ANSYS simulations are also included. The measured cooling rates were ~30°C/s between 94°C and 80°C and ~20°C/s between 94°C and 60°C. The initial cooling of the chip corresponds well to the 2D ANSYS simulation, with the 3D CFD-ACE simulation predicting a slightly faster temperature drop. At lower temperatures there is good agreement between the measurement and CFD-ACE and ANSYS simulations. The measured and simulated values of power consumption and cooling rates are summarized in table 1.

As almost all of the heat is lost by conduction through the substrate, the power consumption needed to keep the chamber at a given temperature is directly related to the thickness and the thermal conductivity of the substrate as well as the area spanned by the heaters. The power consumption of the current design is relatively high, mainly due to the large area of the 20µL chamber. We chose a 20µL chamber so that there would be sufficient volume of sample for post PCR analysis. If high power consumption is a problem, smaller chambers can be fabricated and thicker substrates or substrates with lower thermal conductivity can be used instead of the 1mm thick glass substrate we used. In figure 5 an ANSYS simulation on how the power consumption and cooling rates depend on the substrate thickness is shown. We manufactured the chip on a 1mm glass substrate since this provides a good balance between power consumption and obtainable cooling rates during the thermo cycling. Thinner substrates give faster cooling rates but at the expense of higher power consumption. The cooling rate at the top of the chamber is less depended on the substrate thickness than the cooling rate on the substrate. At a given substrate thickness using a material with higher thermal conductivity than glass will increase both the power consumption and the obtainable cooling rates whereas the use of a material with lower thermal conductivity decreases the power consumption and cooling rates. The substrate can thus be chosen to meet specific needs, as long as the material is compatible with the PCR chamber and electrode fabrication process.

PCR temperature cycling was controlled by a LabView PID controller. In figure 6 a PCR cycle with PID settings that give fast temperature response without overshoot is shown. The transition time from 92°C to 57°C is just under 3s, while the transition time from 57°C to 70°C and from 70°C to 92°C is approximately 1s, with a maximum heating rate of ~50°C/s. As we had expected from the FEM simulations the chip is capable of very fast thermal cycling, with transition times accounting for as little as 5 seconds per PCR themocycle.

To test how accurate the temperature control within the chip is when the integrated temperature sensor is used, we performed a melting curve experiment with *Campylobacter* gene *cad*F PCR product using SYBR green I as fluorescent dye. Melting curve experiments can be used to find the melting temperature T_m of the target DNA, which is the temperature where 50% of the DNA is double stranded while the other 50% of the DNA has denaturated into single strands. Because SYBR green only fluoresces when it is bound to double stranded DNA, the melting temperature

can be found by measuring the fluorescent emission at different temperatures. DNA melting is observed as a sudden decrease in the fluorescence [18]. The melting temperature T_m can also be estimated in a conventional PCR thermocycler by performing PCR denaturation temperature gradient experiments. In these experiments a series of PCR amplification cycles with different settings of the denaturation temperature is performed. Amplification of PCR product will only occur when the denaturation temperature is high enough for melting of the DNA. In figure 7 the results of a melting curve of *cad*F PCR product using the PCR chip and a PCR denaturation temperature gradient experiment using a conventional PCR thermocycler is shown. The melting curve experiment on the PCR chip shows an onset of melting at just under 80^{o}C and that melting is completed at ~85^{o}C. This corresponds to a melting temperature T_m of *cad*F of ~82^{o}C. The PCR denaturation temperature gradient experiments shows amplification of PCR products beginning at a denaturation temperature just over 80^{o}C with maximum PCR product reached at a denaturation temperature of ~85^{o}C. At higher denaturation temperatures the PCR product formation starts to decrease because of increased inactivation of the enzyme. The result of the PCR denaturation experiments corresponds to an estimated melting temperature of *cad*F of ~82.5^{o}C. The good agreement between the melting curve experiments and the PCR thermal gradient experiments shows that the temperature control of the chip is accurate.

Initial testing of the performance of the PCR chamber was performed by amplification of a 199 base pair fragment of yeast ribosomal S3 gene using a hotstart Taq polymerase. In chips both with and without plasma treatment of the SU-8 surfaces we found that PCR amplification was only possible with an increased amount of Taq polymerase (5 units in 20 µl). This is presumably due to absorption of the DNA polymerase and protein on the chamber surfaces. Microfabricated structures in general have a large surface to volume ratio, so absorption to surfaces can cause problems. Due to the high cost of the Taq polymerase, it would be preferable to avoid the need of an increased amount of polymerase. To avoid this we chemically treated the SU-8 surfaces of the PCR chamber with a silanizing agent, dichlorodimethylsilane. The PCR compatibility of the silanized SU-8 chamber was tested by amplification of *Campylobacter* gene *cad*F using standard AmpliTaq DNA polymerase. Using silanized surfaces chips PCR amplification of *Campylobacter* gene *cad*F was possible with only 1 unit Taq polymerase in the 20µL chamber. This is the concentration of Taq polymerase recommended by the supplier. The samples were analyzed using an Agilent bioanalyzer 2100 with a DNA 500 chip kit. In figure 8 two superimposed

electropherograms of the analysis of a PCR amplification of *cad*F done in both silanized chips and in conventional PCR tubes under the same conditions are shown. The peak at ~78s in the electropherogram is the PCR product while the smaller peak at ~90s is an internal marker from the bioanalyser used for calculation of DNA size. The concentration of the PCR amplicon in conventional PCR tubes was ~18 ng/µl whereas PCR in chips gave a concentration of ~12 ng/µl. The yield of PCR product in chips is thus 2/3 of the yield in conventional tubes. Silanization passivates the SU-8 surfaces in the chamber effectively resulting in PCR chips performance comparable to that of conventional PCR tubes.

Conclusion

The PCR chip presented here is the first example of a polymer based PCR chamber with integrated heaters and temperature sensor. The chip demonstrates that SU-8 can be used in the fabrication of PCR microchips. FEM simulations were used in the design of the chip to ensure that the temperature distribution in the PCR chamber is homogeneous and that the chip is capable of fast thermal transitions. Experimental characterization of the chip showed good agreement with the FEM simulations. The excellent thermocycling capabilities of the device with heating and cooling rates in excess of 30^{o}C/s are comparable with the best silicon micromachined PCR chips. The fabrication process of this device is however much simpler than most of the silicon based chips. This can ease on chip integration of PCR with other devices such as CE for post PCR analysis. Amplification of yeast gene ribosomal protein S3 showed that the SU-8 based chamber is PCR compatible if an increased amount of Taq polymerase is used. Silanization of the PCR chamber surfaces resulted in PCR amplication of *Campylobacter* gene *cad*F at the Taq polymerase concentration recommended by the supplier. The performance of silanized chips is comparable to that of conventional PCR tubes.

Acknowledgements

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Biography

Jamil El-Ali received his M.Sc. in Engineering from the Technical University of Denmark (Lyngby, Denmark) in 2000. Jamil is currently a Ph.D. student at the Mikroelektronik Centret. Research interests include design and fabrication of Microsystems for cell and particle handling.

Ivan R. Perch-Nielsen was born in Denmark, 1974, and started at the Technical University of Denmark in 1993. He finished with a M.Sc. in 1999, and started as a Ph.D. in the Cell particle handling group in 2000. Ivans main interests are optics, and optical measurement.

Claus R. Poulsen received his M.Sc. degree in Biology from University of Copenhagen, Denmark, in 1999, working in the field of eletrophysiology. Since 2000, Claus has been working as a Ph.D. student at the Technical University of Denmark, Kgs. Lyngby, on a project integrating PCR to microsystems.

Dang Duong Bang was born in 1951 in Vietnam, received a Biological engineer degree from the University of Hanoi (Hanoi, Vietnam) in 1972. Dang Doung Bang received a M.Sc. in Quality Control Vaccine and Serum from the World Health Organisation (WHO) in 1980 and M. Sc. in Biochemistry from the University of Leiden (Leiden, The Netherlands) in 1990. In 1995, he received his Ph.D. in Molecular genetics from the University of Leiden (Leiden, The Netherlands). In 1996, Dang Duong Bang jointed the Academic Hospital of Maastricht (Maasstricht, The Netherlands) as post-doc in Colon cancer research. Since 1998 he has been employed at the Danish Veterinary Institute (Aarhus, Denmark) as a post-doc researcher and a group leader of the *Campylobacter* pathogenesis.

Pieter Telleman was born in 1965 in The Netherlands, received a chemical engineering degree from the Technical University of Twente (Enschede, The Netherlands) in 1987 and a M.Sc. in Biochemistry from the University of Nijmegen (Nijmegen, The Netherlands) in 1990. In 1995, he received his Ph.D. in Medicine from the University of Amsterdam (Amsterdam, The Netherlands). In 1995, Pieter Telleman joint the Harvard University (Boston, U.S.A.). He joined the Microelectronics Center (Lyngby, Denmark) as a group leader of the Bio/Chemical MicroSystems Group in 1998. In 2000 he assumed the position of research manager at the Microelectronics Center. Pieter Telleman was appointed professor in Bio/Chemical MicroSystems at the Technical

University of Denmark on July 1. 2001. As of September 1. 2002 he is acting director of the Microelectronics Center.

Anders Wolff received a BS from the Danish Engineering Academy (Lyngby, Denmark) in 1991 and a MS in Chemical Engineering from the Technical University of Denmark in 1993. He received his PhD in Biochemical Engineering from Delft University of Technology (Delft, The Netherlands) in 1997. He joined the Microelectronics Center (Lyngby, Denmark) as an assistant research professor in the Bio/Chemical MicroSystems Group in 1998. In 2000 he assumed the position of associated professor and project leader of the Cell Handling Project at the Microelectronics Center.

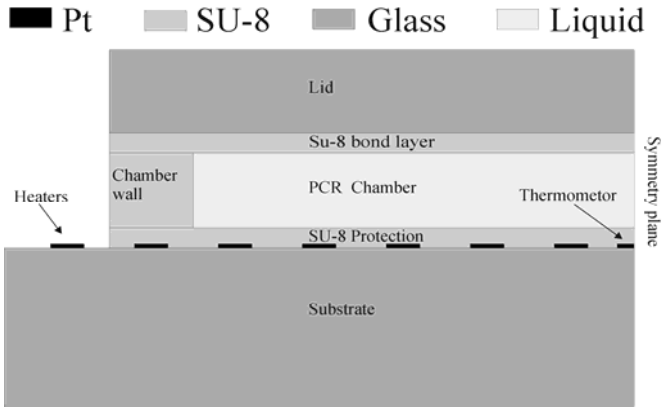

Fig. 1. Schematic illustration of design.

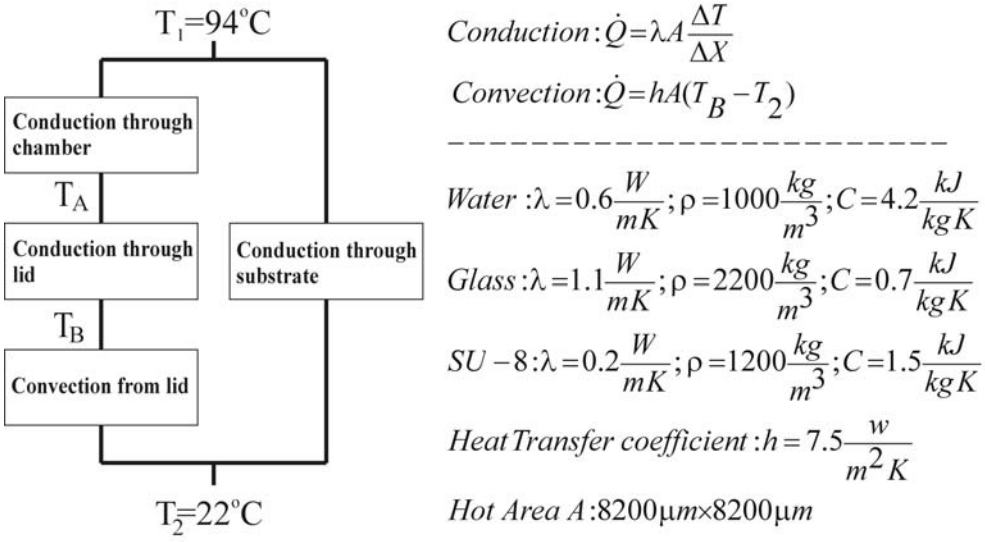

Fig. 2: 1D heat transport mode and material properties used in this model as well as for FEM simulations. λ is thermal conductivity, ρ is density, and C is specific heat capacitance. T_1 is the temperature of the top of the substrate, T_A is the temperature at the top of the chamber and T_B is the temperature at the top of the lid. T_2 is the temperature of the heat sink as well as the ambient temperature.

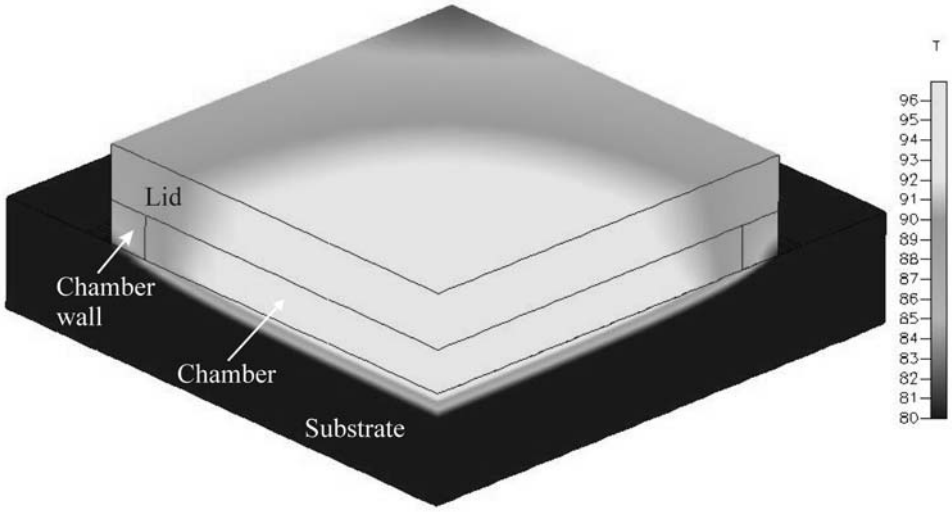

Fig. 3: FEM simulation of steady state temperature distribution at ~94°C. Only one quarter of chip is shown as 2-fold symmetry has been used in the model. The temperature distribution in the chamber is homogeneous with the majority of the chamber is within ±2°C of 94°C.

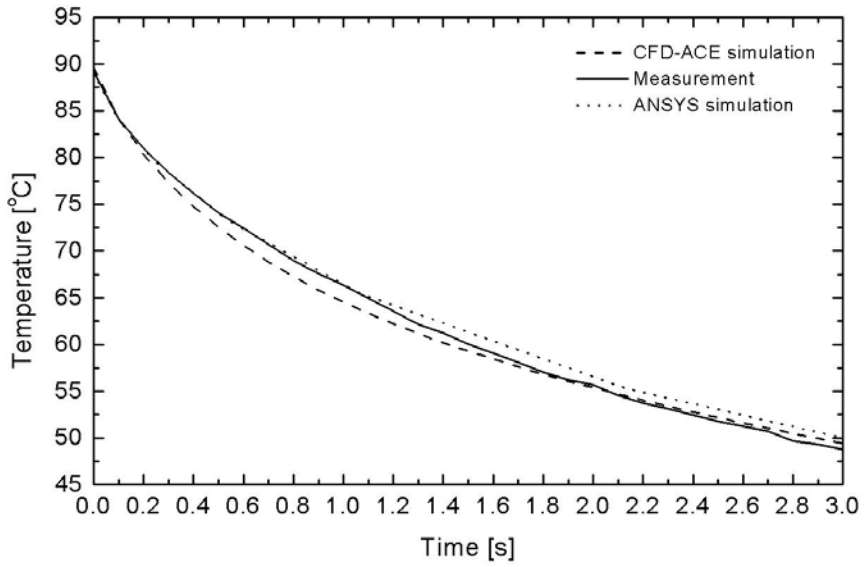

Fig. 4. Simulated and measured cooling of chip, when power is turned off at t=0s.

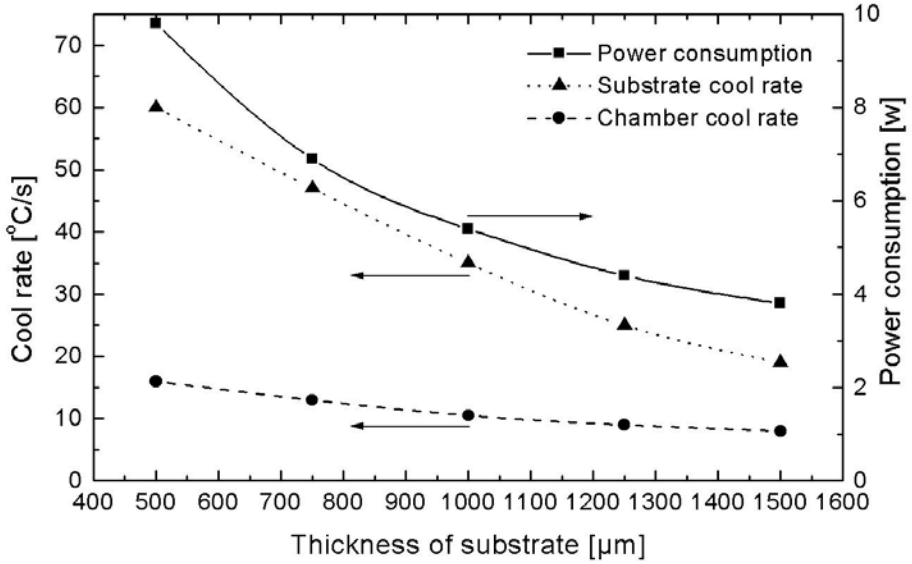

Fig. 5: ANSYS simulation of the dependence of cooling rates and steady state power consumption (94°C) with substrate thickness.

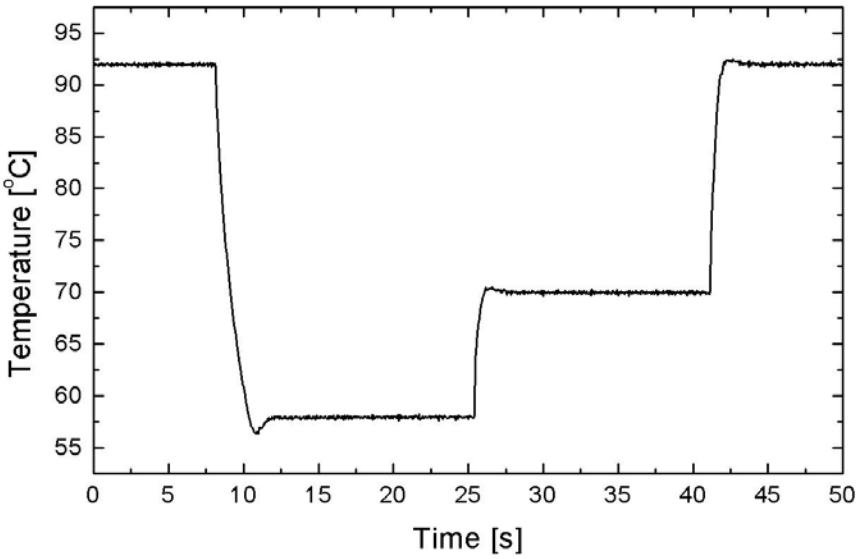

Fig. 6: Temperature response during PID controlled PCR cycle.

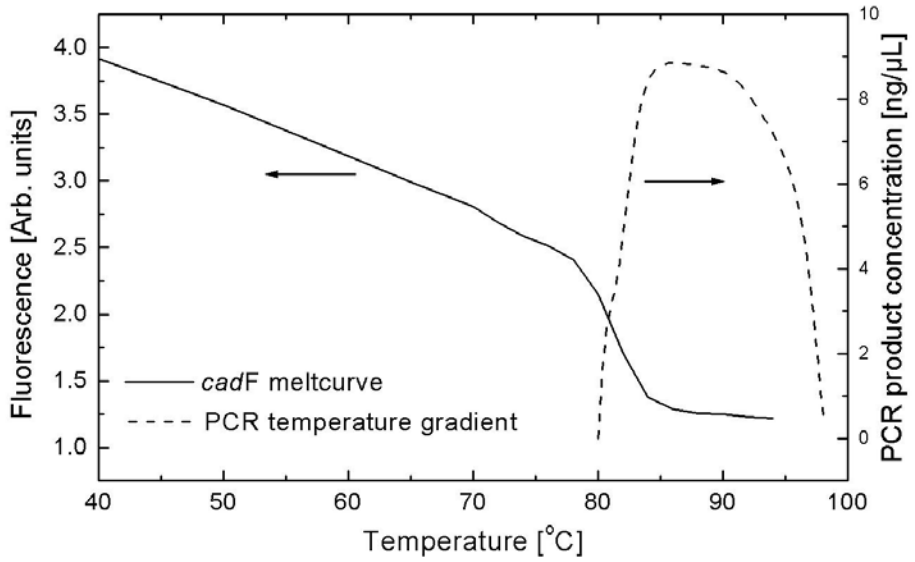

Fig. 7. Melting curve and PCR denaturation temperature gradient experiments of *cad*F PCR product.

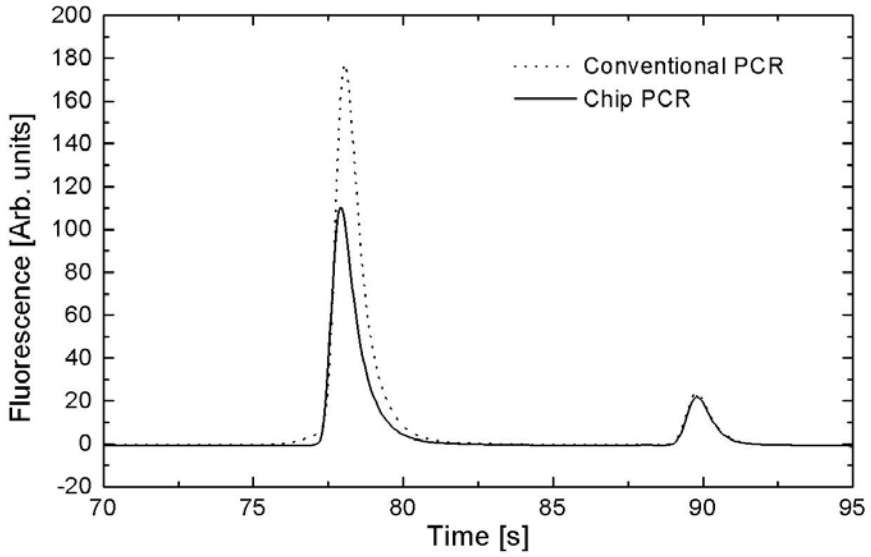

Fig. 8. Electropherogram from Agilent 2100 Bioanalyzer of amplified product from PCR chip and conventional PCR tube.

	ANSYS	CFD-ACE	Experiment
Power consumption@94°C [W]	5.7	5.7	6.3
Max cool rate [°C/s]	~35	~40	~30
Avg. cool rate [°C/s]	~20	~20	~20

Table 1. Simulated and measured steady state power consumption (94°C) and cooling rates of PCR chip.

5 Detection of *Campylobacter* using microfabricated PCR chip (PCR II)

Detection of a putative virulence *cad*F gene of *Campylobacter jejuni* obtained from different sources using a micro-fabricated PCR chip[1]

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Key words: *Campylobacter jejuni*, PCR chip, *Campylobacter* virulence gene, and *cad*F.

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Abstract

AIMS: A micro-fabricated PCR chip made of SU8 was recently designed and developed. In this study, we tested whether the PCR chip could be used for rapid detection of a potential virulence determinat, the *cad*F gene of *Campylobacter jejuni*.

METHODS and RESULTS: PCR was performed using published PCR conditions and primers for the *Camp. jejuni cad*F gene. DNA isolated from a *Camp. jejuni* reference strain CCUG 11284, *Camp. jejuni* isolates obtained from different sources, and *Camp. jejuni* whole cells were used as templates in the PCR tests. Conventional PCR in tube was used as control. PCR positives on chip were obtained from 64% of the tested chips. A fast transition time was achieved with the PCR chip and therefore faster cycling time and a shorter PCR program was obtained. Using the PCR chip, the *cad*F gene could be detected in 1.5 h in comparison to 3.4 h in the published methods.

CONCLUSIONS: The PCR chip can be applied for rapid detection of the virulence *cad*F gene of *Camp. jejuni* obtained from different sources.

SIGNIFICANCE and IMPACT OF THE STUDY: This is the first PCR chip that has been used to detect *Campylobacter* virulence genes. Using the chip the *cad*F gene of *Camp. jejuni* can be detected directly from bacterial whole cells without DNA isolation and purification steps.

Introduction

Campylobacter species are one of the most common causes of diarrhea diseases in humans worldwide (Friedman *et al.*, 2000; Tauxe, 2001). Clinical symptoms of campylobacteriosis in man can vary from mild watery diarrhea to sudden onset of fever, abdominal cramps and diarrhea with blood and leukocytes (Allos *et al.*, 1995). The annual prevalence of *Campylobacter* infections in humans in the United States is estimated to be between 2-8 million cases with approximately 200-800 deaths (Friedman *et al.*, 2000; Tauxe, 2001). An incidence of 86 cases per 100.000 inhabitants in the year 2001 makes *Campylobacter* infection the most common food-borne zoonosis in Denmark (Anonymous, 2002). Large-scale outbreaks of campylobacteriosis in humans are rare and are usually caused by consuming untreated water or raw milk (Harmon *et al.*, 1997; Konkel *et al.*, 1999). Sporadic cases are usually caused by ingestion of *Campylobacter* infected foods or food products. Poultry and poultry products are considered to be the major source of infection in human campylobacteriosis (Deming *et al.*, 1987; Friedman *et al.*, 2000; Harris *et al.*, 1986).

Campylobacter carriage in broiler flocks has been reported from many countries (Berndtson *et al.*, 1996; Jacobs-Reitsma *et al.*, 1994; Kapperud *et al.*, 1992). In Denmark, the prevalence of *Campylobacter* infected broiler flocks at slaughter has recently been reported to be 37.7% (Wedderkopp *et al.*, 2000).

Despite *Camp. jejuni* being one of the major causes for gastro-enteritis in humans worldwide, the details for the molecular pathogenesis are not well understood (Wassenar, 1997). Several virulence determinants have been proposed for *Campylobacter* including flagella–mediated motility, adherence to intestinal mucus, invasion capacity, and the ability to produce toxins (Ketley, 1997; Wassenar, 1997). The genome sequence of *Camp. jejuni* has recently become available and the development of a number of in vitro assays has enabled molecular identification and characterization of *Campylobacter* virulence and toxin genes. Among these genes, a 37kDa outer membrane protein named CadF (short for *Campylobacter* Adhesion to Fibronectin) has been identified as a *Campylobacter* adhesin (Konkel *et al.*, 1997). Immunoblot analysis using rabbit anti *Camp. jejuni* CadF 37 kDa serum has shown that the *cad*F gene and its products are conserved among a number of *Camp. jejuni* isolates (Konkel *et al.*, 1999). Recently, the prevalence of the *cad*F gene among *Campylobacter* isolates obtained from different sources has been reported (Bang *et al.*, 2003). Furthermore, the involvement of the *cad*F gene products in *Campylobacter* colonization has clearly been shown by in vivo colonization experiments using wild types and

mutants of *Camp. jejuni* strains, where the *cad*F gene was disrupted via homologous recombination (Ziprin *et al.*, 1999).

Over the past decade, micro-fabricated chip technology has become an area of huge interest. Recently, a micro-fabricated PCR chip has been designed and developed (El-Ali *et al.*, 2003). In this study, we tested whether the newly developed PCR chip can be applied to detect the *cad*F gene of *Camp. jejuni*. PCR on chip was tested using the published *cad*F gene primers with purified DNA from *Camp. jejuni* reference strain CCUG 11284 and from a number of *Camp. jejuni* isolates obtained from different sources. The successfulness of the PCR on chip using different targets (purified DNA, bacterial whole cells) and the influence of PCR conditions (number of cycles, PCR programs) on the PCR yields will be presented and discussed.

Materials and Methods

Chip fabrication

The SU-8 PCR chamber was manufactured on a 1mm thick glass substrate with thin film platinum heaters and temperature sensor integrated on chip for thermo cycling. The chamber dimensions on the PCR chip were 7mm x 7mm x 0.4mm resulting in a chamber volume of 20µL. The polymer based PCR chamber is fabricated as described previously (El-Ali *et al.,* 2003). Briefly, thermometer and heater electrodes were patterned by lift-off in a 2000Å platinum layer on the glass substrate. The electrodes were protected from PCR mixture by a 5 µm SU8 layer (XP2005, MicroChem, MA, US) patterned by photolitography. The PCR chamber walls were defined in a 400µm SU-8 layer (XP2075, MicroChem, MA, US). The 400 µm SU-8 layer was achieved using a multi spin procedure: two spins of 200µm, with a soft-bake step after each spin. The glass wafer was finally diced into PCR chips.

Chip coating

To avoid binding of PCR materials to the chip surface all chips were coated by a method previously described (Sambrook *et al.*, 1989) with modifications: Dichlorodimethylsilane (Merck-Schuchardt, Hohenbrunn, Germany) was vapor deposited at partial vacuum in a desiccator for one hour. Subsequently the chips were washed twice in ultra pure sterile filtered water for 15 minutes and finally once for 20 minutes. The chips were dried with compressed nitrogen and stored in a dry place at room temperature until use.

Operating the PCR chip

Prior to use, the chip was placed in an aluminum holder that also acted as a heat sink. The PCR chamber was covered by a lid (Fig. 1). The lid had a 300µm layer of PDMS (Sylgard 184; Dow Corning, Michigan, US) on a support of 1mm PMMA plastic (RIAS A/S, Roskilde, Denmark). The PCR temperature cycling was controlled by a LabVIEW based PID controller (National Instruments, Austin, TX, USA). Cooling of the chip was achieved passively by heat conduction through the substrate to the heat sink or by natural convection from the lid.

Bacterial culture, DNA extraction, and dilution series

The chromosomal DNA and the whole cell of a bacterial reference strain *Camp. jejuni* CCUG 11284 was used as PCR templates. The bacterial culture conditions are described elsewhere (Bang *et al.,* 2003). A bacterial overnight culture on a blood agar plate (BA plate; Blood agar Base no. 2, Oxoid, supplemented with 5% sterile defibrinated calf blood) grown under microaerobic

conditions (6% O_2, 6% CO_2, 4% H_2 and 84% N_2) at 42 °C was used to prepare the bacterial chromosomal DNA. The bacterial chromosomal DNA was extracted according to the method previously described (Bang *et al.*, 2001). The DNA was eluted in 100µl sterile water preheated to 65 °C. The DNA was stored at –20 °C until use.

For PCR experiments, using bacterial whole cell as PCR templates, *Camp. jejuni* cells was prepared from a overnight culture on BA plate of *Camp. jejuni* reference strain (CCUG 11284) as described above. A loop full of bacteria was thoroughly suspended in 500µl sterile filtered de-ionized water. The initial cell concentration in this suspended solution was $14x10^8$ cells ml^{-1}. A dilution row was made from the freshly harvested bacteria.

Primers and PCR conditions

Two primers, F2B and R1B of the *cad*F gene previously described (Bang *et al.*, 2003; Konkel *et al.*, 1999) were applied in a PCR to detect the *cad*F gene of *Camp. jejuni* using the micro-fabricated PCR chip. All PCR mixtures (25µl) containing 1x Taq DNA polymerase buffer and 0.05 U $µl^{-1}$ Taq DNA polymerase (Boehringer-Mannheim, Germany), 0.625 mM $MgCl_2$, 0.25 mM of each dNTP, 200 fM of each primer (DNA Technology, Aarhus, Denmark). Conventional PCR in tubes (0.2 ml thin-walled thermo-tubes, ABgene, Surrey, UK) were performed in parallel on all the samples as controls and the PCR was performed in a thermocycler PTC-200 (MJ Research, Inc., MA, US). The PCR conditions used in this study were similar to those previously described (Bang *et al.*, 2003; Konkel *et al.*, 1999), i.e. 94°C for 5 min followed by 40 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 3 minutes. The final step was 72°C for 10 min. However, modified PCR programs were also used. The modified programs had 5 initial cycles as described above followed by 35 or 25 cycles with reduced denaturation, annealing and elongation time as described in the results.

DNA analysis

All PCR products were analyzed on the microchip based Agilent 2100 Bioanalyzer (Agilent Technologies, CA, US) using the DNA 500 kit. The DNA 500 kit can analyze DNA fragments up to a length of 500 base pairs.

Results

Detection of the Campylobacter jejuni cadF virulence gene using both conventional PCR tubes and the micro-fabricated PCR chip

A total of 25 micro-fabricated PCR chips were used to test whether the PCR chip can be applied to detect the *cad*F gene of *Camp. jejuni*. DNA extracted from pure culture of *Camp. jejuni* reference strain CCUG-11284 was used as template. Conventional PCR in tube was included as control. Of 25 chips tested, 16 (64%) were PCR positive, whereas all conventional PCR in tubes were positive (Table 1).

Detection of the cadF gene in DNA preparations extracted from various sources.

It was tested whether the PCR chip could be applied with the DNA templates prepared from various sources: DNA templates prepared from *Campylobacter* isolates obtained from different sources included a *Camp. jejuni* reference strain (CCUG 11284), two *Camp jejuni* human isolates (human 679, human 682) obtained from fecal material of patients with campylobacteriosis, and a *Camp. jejuni* chicken isolates (chicken SC-445) originating from chicken fecal sample at slaughter were used as templates in PCR both on chips and tubes. Results of such experiments are summarized in Table 2. Positive PCR detection of the *cad*F gene was obtained in all the samples. However, results of analysis PCR products using the microchip based Agilent 2100 revealed that the concentrations of PCR product of PCR on chips with the reference strain and the human isolates were 2 fold lower compared to conventional PCR on tubes. No difference was observed with the chicken isolate (Table 2).

Detection of the cadF gene using Campylobacter whole cells as DNA template Results of PCR assays in which *Camp. jejuni* whole cells were used as template are illustrated in Fig. 2. PCR amplicons with expected size of 400 bp were observed at cell concentrations of 56, 560, and 5600 cells μl^{-1}. The DNA concentration of PCR products were determined to be 2.7±0.1 nmol l^{-1}, 14.6±0.2 nmol l^{-1}, and 2.6±0.2 nmol l^{-1}, respectively (Fig. 2). PCR negative was obtained at both higher and lower cell concentrations. The optimal cell concentration was determined to be 560 cells μl^{-1}. This cell concentration was used in further experiments with PCR on chip. Table 3 represents results of 3 PCR chips of such experiments. The DNA concentrations of the PCR products were on average17.7 ±2.8 nmol l-1 with PCR-chips and 29.4 ±1.9 nmol l-1 with conventional PCR on tubes.

Determination of a PCR program suitable for the micro-fabricated PCR chip.

The optimal reaction time for the PCR chip was determined by two ways: 1) decreasing the time for each PCR cycles, and 2) by decreasing the number of cycles. Results of PCR on chip analyses performed with a decreased reaction time for each PCR cycle are presented in Table 4. The total time for each cycle that included denaturation (d = 1 min), annealing (a = 1 min), and elongation time (e =3 min) was initially of 5 min exclusive heating and cooling times. When reducing the time for each cycle from 5 min to 2 min, the yield of PCR chips remained about 45 nmol l^{-1}, whereas in the tubes, the yields were reduced by 1.7 fold (Table 4). Further reduction of the time of each cycle from 5 min to 1.5 or 1.3 min reduced the PCR yields in the chips by 3.0 – 3.7 folds, whereas in the tubes PCR yields were reduced 2.3 - 2.6 folds. A suitable time of each cycle of 2 min was selected and used as an optimal cycle time to set up the PCR-chip program.

Table 5 represented the results of running this PCR chip program at 30 and 40 cycles, respectively. It was revealed that reducing the number of cycles from 40 to 30 reduces the PCR yield by 4 fold (Table 5).

Discussion

Several factors can influence the PCR yield on chip such as the materials used to produce the chip, silanization of the inner surface of the chip, heating and cooling system, DNA target, Taq polymerase etc. However, the silanization of the inner surface of the PCR chip is one of the essential factors for the result of PCR on chip. In initial experiments when Taq polymerase concentration was low (0.01 U μl^{-1}) in comparison to the concentration used for conventional PCR in tubes (typically 0.025 U μl^{-1}), no PCR positives were obtained on chip, while all the conventional PCR in tubes were positive. Positive results with the PCR chip were obtained when a higher concentration of Taq polymerase (0.05U μl^{-1}) was used. This concentration was therefore applied in both PCR chip and in conventional PCR in tubes thoughout the remainder of the study. We found that the yields of the PCR-chip varied from experiment to experiment. Similarly, Giordano *et al*. (2001) reported a variation of the positive results with their PCR on chip due to an inadequate surface coating resulting in the adsorption or inhibition of Taq polymerase. Shoffner *et al.* (1996) showed that a better and more efficient silanization of the PCR chips generated PCR results comparable to the results obtained by PCR in tubes (Shoffner *et al.*, 1996). In our case 64% of the PCR chips tested PCR positive (Table 1) and positive PCR chip were only obtained when higher concentration of Taq polymerase was used. This most probably reflects the absorption of Taq polymerase and the inhibition effects of the inadequate coated inner surface of the chip. Therefore, a standardized method for coating the inner surface of the PCR chip should be addressed in future works.

Using the PCR chip, the *cad*F gene was detected in all four *Camp. jejuni* isolates tested. The templates used for these PCR chips were purified DNA preparations. The PCR yield in PCR chips represented by DNA concentration (nmol l^{-1}) of the PCR products determined by a commercial kit (Agilent 2100 Bioanalyzer with DNA 500 kit) was from 2.4 fold lower to equal in comparison to the conventional PCR in tubes as control (Table 2). For PCR assays, performing of sample pre-treatment and DNA extraction are laborious and time-consuming procedures. It would therefore be advantageous to be able to perform PCR directly on *Campylobacter* cells. A dilution row of *Campylobacter* cells (0.56 to 56.000 cells μl^{-1}) was used as templates in a conventional PCR in tubes to determine the whether the *Camp. jejuni* whole cells could be used directly as template. The result of the experiment revealed that PCR negative were obtained both in too high (>5600 cells μl^{-1}) or too low (<56 cells μl^{-1}) cell concentrations, and that the most suitable cell

concentration for PCR was 560 cells μl^{-1} as reflected by the highest DNA concentration of the PCR product (14.4 nmol l^{-1}). Interestingly, applying this cell concentration on PCR chips, PCR positive results were obtained in 100% (3/3) of the PCR chips tested. The fact that PCR negative reactions were obtained with too low cell concentration (<56 cells μl^{-1}) can be explained simply by stating that there is not enough DNA template for the PCR. However, PCR negative was also obtained with too high cell concentration (>56.000 cells μl^{-1}). This could probably be due to several reasons: 1) Cell lysate or cellular debris from broken cells caused by the heat-denaturing step in PCR could inhibit the Taq polymerase, 2) cell lysate or intracellular compounds released from broken cells could act as inhibitors of the PCR, or 3) cell lysate or cellular components of the broken cells could change the concentration of specific ions in the PCR solution.

One of the advantages of the PCR chip is the possibility of getting a shorter time of PCR assays. A shorter PCR program could be achieved in two ways: 1) by shortening the time of each cycle or 2) by reducing the number of PCR cycles. The PCR chip was designed with a low thermal mass. Therefore, faster rates of heating (50°C s^{-1}) and cooling (30°C s^{-1}) in comparison to 3°C s^{-1} in a conventional system (e.g. MJ Research PTC-200 DNA engine) were achieved (El-Ali *et al.*, 2003). A reduction of the total time (denaturation, annealing and elongation time) of each PCR cycle from 5 min to 2 min had no effect on the PCR yields. However, when the time was reduced to 1.5 or 1.3 min the PCR yields were reduced 3.0 – 3.7 folds (Table 4). Successful PCR on chip with a cycle time of 1.3 min as presented here is much shorter in comparison to 5 min of each cycle in a conventional PCR to detect the *cad*F gene of *Camp. jejuni* in previous studies ((Bang *et al.*, 2003; Konkel *et al.*, 1999). It has been suggested that in the PCR, the denaturation of DNA strands and the annealing of primers to the DNA target might occur instantaneously (Oda *et al.*, 1998; Wittwer *et al.*, 1990). Therefore, a shorter time for each PCR cycle of 20 sec (Wittwer *et al.* 1990), of 8 sec (Oda *et al.* 1998) or of 2 sec (Giordano *et al.* 2001) in other studies have been reported. A number of publications have suggested that lowering the cycle time would improve the overall yield of the amplification (Huhmer *et al.*, 2000; Wittwer *et al.*, 1990; Wittwer *et al.*, 1989). Fast transition times favouring primer annealing will improve PCR efficiency, and decreasing the heat denaturation time will reduce the inactivation of Taq DNA polymerase, which in turn will lead to a higher overall enzymatic activity in the reaction. However, in this study, decreasing the time of each cycle did not improve the PCR yields (Table 4). Furthermore, increasing the number of PCR cycles from 30 to 40 did increase the yield on PCR chip 4 folds (Table 5).

In conclusion, in the present study we showed that the PCR chip could be applied to detect the virulence *cad*F gene of *Camp. jejuni* isolates obtained from human and poultry. With the advantage of the PCR chip having a faster transition time, a shorter time of 1.3 min for each PCR cycle was achieved. Using the PCR chip the *cad*F gene of *Camp. jejuni* could be detected within 1.5 h in comparison to 3.4 h of a conventional tube-PCR previously described . Interestingly, using the PCR chip, it was possible to detect the *cad*F gene directly from the whole cells without any DNA purification step that is laborious and time-consuming. To our knowledge this is the first time a PCR chip was used to detect a virulence determinant gene of *Campylobacter*. The development of a multiplex PCR chip for simultaneously detecting several virulence and toxin genes of *Campylobacter* are in progress.

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Table 1. Success rate of PCR in microchip. A total of 25 experiments were performed in microchip with corresponding identical control experiments made in conventional tube-PCR.

PCR results	PCR on tube	PCR on chip
Negative (%)	0	9 (36)
Positive (%)	25 (100)	16 (64)
Total (%)	25 (100)	25 (100)

Table 2. Amplification of *Campylobacter jejuni* DNA from various sources.

Isolate	Chip nmol l^{-1}	Tube nmol l^{-1}	Ratio chip/tube %
CCUG 11284	41.5	72.2	57
Human 679	33.5	80.4	42
Human 682	62.0	90.4	69
Chicken SC-445	21.8	22.2	99

Table 3. Concentration of PCR product from PCR with whole *Campylobacter jejuni* cells as template.

Experiment no.	Chip nmol l^{-1}	Tube nmol l^{-1}	Ratio chip/tube %
1	20.1	30.4	66
2	18.0	30.4	59
3	14.9	27.5	54

Table 4. Concentration of PCR product with different cycle times. Total cycle time and denaturation, annealing, and elongation time (d, a, e, respectively)

Cycle time Total (d, a, e)	Chip nmol l^{-1}	Tube nmol l^{-1}	Ratio chip/tube (%)
5min (1min, 1min, 3min)	41.5	72.2	57
2min (30sec, 30sec, 1min)	41.5	42.5	98
2min (30sec, 30sec, 1min)	55.7	42.5	131
1.5min (15sec, 15sec, 1min)	13.2	27.3	48
1.5min (15sec, 15sec, 1min)	14.8	27.3	54
1.3min (10sec, 10sec, 1min)	11.3	31.0	37

Table 5. Concentration of PCR product with different number of PCR cycles.

No. of Cycles	Chip nmol l^{-1}	Tube nmol l^{-1}	Ratio chip/tube (%)
30	11.5	71.9	16
30	13.1	71.9	18
40	41.5	42.5	98
40	55.7	42.5	131

Fig. 1. Schematic of the PCR chip with PDMS bonded lid. The PCR chip is placed in a recess in an aluminium block, which acts as a heatsink. Pressure to the PDMS bonded lid is provided via a PMMA pressure support plate using spring-mounted screws. Electrical connection is made through pogo pins positioned in the PMMA support plate and aligned to bond pads on the chip.

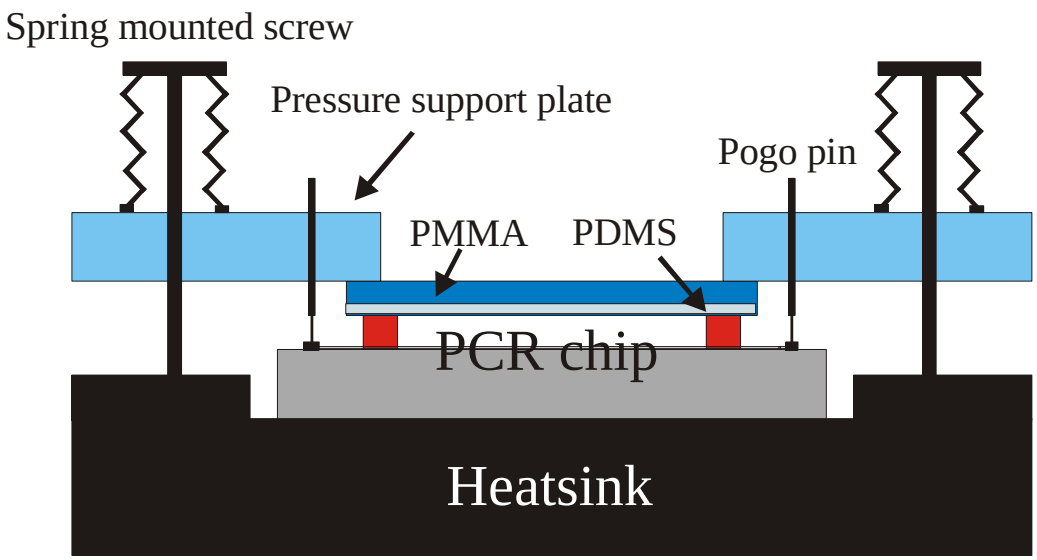
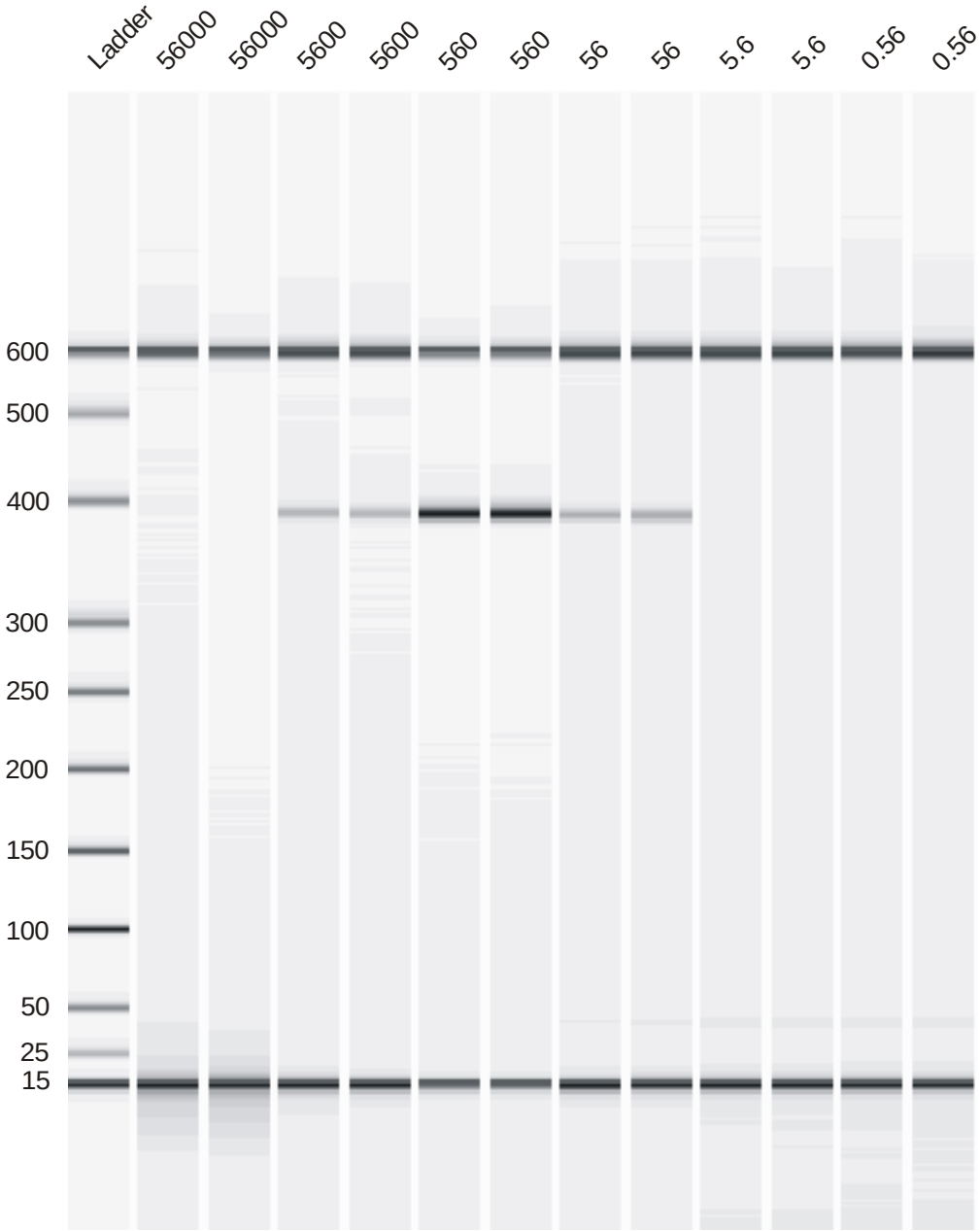


Fig. 2. Dilution series of *Campylobacter jejuni*. Dilutions range from 0.56 to 56000 cells μl^{-1}. The size of the ladder bands (in bp) is written to the left.



6 Sample pretreatment

Removal of PCR inhibitors using dielectrophoresis as a selective filter in a microsystem[1]

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Abstract

Diagnostic PCR has been used to analyse a wide range of biological materials. Conventional PCR consists of several steps such as sample preparation, template purification, and PCR amplification. PCR is often inhibited by contamination of DNA templates. To increase the sensitivity of the PCR, the removal of PCR inhibitors in sample preparation steps is essential and several methods have been published. The methods are either chemical or based on filtering. Conventional ways of filtering include mechanical filters or washing e.g. by centrifugation. Another way of filtering is the use of electric fields. It has been shown that a cell will experience a force when an inhomogeneous electric field is applied. The effect is called dielectrophoresis (DEP). The resulting force is depending on the difference between the internal properties of the cell and the surrounding fluid. DEP has been applied to manipulate cells in many microstructures. In this study, we used DEP as a selective filter for holding cells in a microsystem while the PCR inhibitors were flushed

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out of the system. Haemoglobin and heparin – natural components of the blood – were selected as PCR inhibitors, since the inhibitory effects of these components to PCR have been well documented. The usefulness of DEP in a microsystem to withhold baker's yeast (*Saccharomyces cerevisiae*) cells while the PCR inhibitors haemoglobin and heparin are removed will be presented and factors that influence the effect of DEP in the microsystem will be discussed. This is the first time dielectrophoresis has been used as a selected filter for removing PCR inhibitors in a microsystem.

Introduction

Polymerase chain reaction (PCR) has been widely used to detect microorganisms in clinical diagnostic and food industry. However, when conducting PCR on complex biological materials (blood, milk, faeces etc.), residual materials from the samples or from pretreatment steps can inhibit and significantly reduce the efficiency of the PCR process [1, 2]. The mechanism of the inhibition can e.g. be a competition between inhibitor and DNA template or chelation of the ions used in the process [3]. It has been well documented that the PCR inhibition can be solved by selecting a suitable method for pretreatment the sample, by selection of a suitable method to isolate DNA templates**,** or by selecting a DNA polymerase that is less affected by the inhibitor [4]. Sample pretreatment is an essential step to limit the PCR inhibition effects. Pretreatment is either chemical or mechanical [5]. Filtering or washing by centrifugation can be used to wash away the inhibitors as long as the inhibitors do not bind to the cells or are not inside the cells, but both methods are laborious and are not suitable for microsystems. In addition, filters holding the cells can get clogged or collect air, and the cells tend to stick on the surface. Dielectrophoresis (DEP) [6, 7] has previously been used for manipulation and sorting of cells in microsystems [8-10]. In this paper we describe a new approach to use of DEP as a selective filter in a DEP chip to remove PCR inhibitors. By using DEP it is possible to attach the cells to electrodes, while other materials with different dielectric characteristics (in this case; inhibitors) are passing unhindered through the system. Interestingly, when using DEP as selected filter in the DEP chip, the DEP force can be turned off and the cells released as a ready sample for the PCR.

Principle of the Dielectrophoresis (DEP)

When an electric field is applied upon particles such as biological cells, a dipole is induced [11, 12]. If the electric field is non-uniform, a part of the dipole will experience a stronger field, thereby creating an overall force on the cell. This force is not dependent on the polarity of the external field, but on internal properties of the cell compared to its surroundings. The time average of the DEP force on a spherical cell can be written as

$$\left\langle F_{dep} \right\rangle = 2\pi\varepsilon_f R^3 \,\mathrm{Re}[\underline{K}(\omega)]\nabla E^2 \qquad (1)$$

Where ε_f is the permittivity of the fluid, R is the cell radius, E the electric field strength and $\underline{K}(\omega)$ is the complex Clausius-Mossotti factor, which is the strength of the effective polarization of the cell. With the electrode geometry in our system**,** the direction of the force is defined purely by the Clausius-Mossotti factor, which is

$$\underline{K}(\omega) = \frac{\underline{\varepsilon}_c - \underline{\varepsilon}_f}{\underline{\varepsilon}_c + 2\underline{\varepsilon}_f} \qquad (2)$$

Here $\underline{\varepsilon}_c$ and $\underline{\varepsilon}_f$ is the complex permittivity of the cell and fluid, respectively. The complex permittivity is defined as $\underline{\varepsilon} = \varepsilon - i\sigma/\omega$, where $i = \sqrt{-1}$ and $\omega = 2\pi f$ is the radial frequency of the imposed electrical field. σ is the conductivity of the current media (σ_c for a cell and σ_f for fluid) and ε is the permittivity.

To illustrate one important aspect of the system**,** whether cells are attracted or repelled from the electrodes, it is useful to rewrite $\underline{K}(\omega)$ in terms of the characteristic relaxation time constants [11]

$$\underline{K}(\omega) = \frac{\sigma_c - \sigma_f}{\sigma_c + 2\sigma_f} \cdot \frac{i\omega\tau_o + 1}{i\omega\tau_{mw} + 1} \qquad (3)$$

Where $\tau_o = (\varepsilon_c - \varepsilon_f)/(\sigma_c - \sigma_f)$ and $\tau_{mw} = (\varepsilon_c + 2\varepsilon_f)/(\sigma_c + 2\sigma_f)$. The combining of equation 1 and 3 reveals that when the conductivity of the fluid is higher than the effective conductivity of a cell, the cell will experience a negative DEP force and be repelled from the electrode (negative DEP). When σ_f is lower than σ_c the cell will be attracted to the electrodes (positive DEP). To ensure the cells always experience a positive DEP force σ_f must be lower than σ_c. For the experiments described in this study sterile water with lower conductivity is always used for washing. The cells attached to the electrodes in the beginning of a washing process are therefore not released during the washing and knowing the exact value of σ_c is not important.

Materials and Methods

Fabrication of the microsystem

The microsystem was a DEP chip fabricated on a silicon substrate. A 2 µm LPCVD (Low Pressure Chemical Vapour Deposition) nitride layer was deposited on the silicon substrate to insulate the electrodes from the substrate. On top of that a 180 µm LPCVD polysilicon layer was deposited. An 80 nm layer of titanium was deposited using E-beam evaporation and structured using a lift of process. To remove the natural oxide a 30 second HF (hydrofluoric acid) dip was applied just before titanium deposition. The silicide was formed using rapid thermal annealing for 1 minute at above 700°C in an argon atmosphere. The electrode design was an interdigitated-finger structure [6] with a characteristic dimension of 10 µm and a length of 2 mm (figure 1). The walls of the channel were made of SU-8 [13] and the channel was height × width = 70 × 400 µm. The total channel length in the microstructure was 11 mm.

Yeast cells

The baker's yeast (*Saccharomyces cerevisiae*) (Danisco A/S, Denmark**)** cell was chosen as a model in the experiment. The yeast cells were grown overnight at 37 °C in Yeast extract Nitrogen Base (YNB) medium supplemented with 40% glucose and 40 µg/l histidin (Bie & Berntsen A/S, Denmark). Cells were collected by centrifugation, and the medium was removed. The cell pellet was washed three times in 1 ml water (ultra-clean water, MERCK eurolab). One hundred µl of the cell suspension (10^7 cells/ml) were collected, and used as positive PCR controls (without PCR inhibitor).

Inhibitors

Heparin (Sigma, St. Louis, Missouri, USA) and bovine haemoglobin (Sigma, St. Louis, Missouri, USA) were selected as inhibitors for testing the ability to remove inhibitors from the cells using the DEP microstructure, since both heparin and haemoglobin are well known as PCR inhibitors. One hundred µl of a solution with the inhibitors in 10× the final concentration were added to 900 µl of the cell samples. This gave a final concentration of 1.3 mg/ml for heparin (giving a conductivity of $\sigma_f = 23$ mS/m) and 10 mg/ml for haemoglobin (giving $\sigma_f = 52$ mS/m), 30 µl of this mixture – containing both cells and the chosen inhibitor – was used as PCR controls. The final concentration of inhibitors was made several orders of magnitude higher than the concentration that gives PCR inhibition effect [3]. The mixture of yeast cells with inhibitors were prepared and kept at room temperature for 1 hour before use.

Preparing the DEP chip for experiments

The DEP chip was cleaned thoroughly before use. Firstly, the DEP chip was flushed through with sterile water, then with ethanol followed by sodium dodecyl sulphate (SDS) 10%, and finally with sterile water to remove any residues of SDS. The system was then ready for use.

Primers and PCR conditions

Two primers, namely Ribo.Prot.S3-forward and Ribo.Prot.S3-reverse were used in a PCR to amplify the yeast ribosomal protein S3 – a housekeeping gene. The Ribo.Prot.S3-forward primer was a 21-mer primer with the sequence 5'-AAT CCT CAG GCA AAT GTA AAA-3'. The Ribo.Prot.S3-reverse primer was a 20-mer primer with the sequence 5'-CTT AGG CAA ATC AAA AGC AT-3' (TAG Copenhagen A/S, Denmark). PCR amplicons of 199 bp of the yeast ribosomal protein S3 gene were generated in a PCR with these primers.

All PCR mixtures (20 µl) contained 1× AmpliTaq Gold PCR Master Mix (Applied Biosystems, CA, US) and 0.156 µM of primers. The PCR was performed in a thermocycler (PTC-200; MJ Research, Inc., MA, USA) for 35 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 1 min after a hot start at 94°C for 10 min. The last step was an extension at 72°C for 10 min. Ten µl of yeast cells suspension was used as templates. Yeast cells in pure water were used in PCR positive controls, water in negative controls, and yeast-inhibitors (heparin or haemoglobin) in inhibition controls.

DNA analysis

All PCR products were analysed on the microchip based Agilent 2100 Bioanalyzer (Agilent Technologies, CA, US) using the DNA 500 kit, which can analyse DNA fragments up to a length of 500 bp.

Results

Using the DEP chip as selective filter to remove PCR inhibitor

A schematic overview of an experimental set up using the DEP chip to wash away the inhibitor from the yeast cells is presented in Figure 2. For initialisation of the chip, the DEP chip was filled with water from a syringe used for washing. Details of the 3-step of manipulation of the DEP chip are illustrated in Figure 3. In step 1, the withdrawing syringe was started, thereby sucking the sample into the channel. A voltage of 20 V_{pp} at 1 MHz was applied to the electrodes and the yeast was collected on the electrodes by positive DEP. After $1-2$ min (depending on density of cells), the electrodes were saturated with yeast cells, the suction was stopped, and the sample container vial was removed from the structure (Figure 3, step 1). In step 2, pure water was flushed through the channel with a flow rate of 0.2 ml/h (figure 3, step 2). After a selected time (0 to 30 minutes), step 3 was started. The voltage applied to the electrodes was turned off, the cells were released, and the pump was set to 1 ml/h to flush out the cells (Figure 3, step 3). One drop (approximately 20 µl) of the released cells was collected from the output for PCR analysis. Between each run in an experiment with the same inhibitor, the DEP chip was flushed with clean water by setting the syringe pump with a speed of 3 ml/h for few min.

The influence of different factors on the efficiency of the DEP chip

DEP force

When attracted to the electrodes, the cells were lying side by side, forming a thick layer that might be several times thicker than the cell diameter. The electric field strength decreases with the distance from the electrodes, and cells at the top of the layer were therefore exposed to a lower DEP force and more sensitive to the flow. Applying a low flow-rate and a high field-strength will maximize the amount of collected cells.

Conductivity

To determine the highest effective conductivity that is applicable for the DEP chip, a dilution row of KCl in water was made and mixed with the yeast cells and the mixture was pumped through the DEP chip. We determined that with the flow set to 0.2 ml/h and a field of 20 V_{pp} at 1 MHz, yeast cells could be collected when conductivity reached up to 140 mS/m, but at very low efficiency. A useable conductivity of the sample applicable in our DEP chip using the yeast cell was determined to be 70 mS/m.

Flow rate

The flow rates of the DEP chip were varied from f = 0.1 ml/h to 1.0 ml/h. A syringe pump manually controlled the flow rate. With a cross-section of the structure, A = 400 μm × 70 μm, the flow rate was calculated to have an average flow velocity ($v = f/A$) around 1 mm/s – 10 mm/s. We also found that applying a higher velocity reduced the amount of collected cells.

Determination of PCR inhibitor effect of haemoglobin and heparin

The effect of haemoglobin and heparin on the PCR amplification of the yeast ribosomal protein S3 gene was investigated. Yeast cells and haemoglobin or heparin were mixed and 10 μl of this sample was used as template (Table 1). These initial experiments showed that the PCR reactions were completely inhibited by a haemoglobin concentration in the sample of 1 mg/ml and by a heparin concentration of 13 μg/ml.

Effects of DEP chip to remove the PCR inhibitor effect of heparin and haemoglobin

The result of PCR amplifications of the yeast ribosomal protein S3 gene using the yeast cells washed in the DEP chip as template is presented in Table 2. The yeast cells in water (10^7 cells/ml) were mixed with haemoglobin (10 mg/ml), or heparin (1.3 mg/ml), respectively, and the cell/inhibitor mixtures were applied to DEP chip for washing as described above. The cells were collected after washing 2, 5, 7.5, 10 or 30 min, respectively. The cells were used directly as PCR templates. A 199 bp PCR amplicon of the yeast ribosomal protein S3 gene was observed in all the PCR in which the yeast cells collected after 10 or 30 min. washing were used as templates. With haemoglobin, positive PCR results were obtained for samples collected after 5 min. of washing while with heparin, positive PCR were only observed with samples collected after 10 min of washing. We also found that with the flow rate of 0.2 ml/h, a sufficient washing time was approximately 10 min.

Discussion

In this study, the baker's yeast *S. cerevisiae* cell was used as a model organism for investigating the capability of dielectrophoresis in a microsystem to remove inhibitors from the cells. Yeast were chosen for several reasons: It is unicellular, safe to handle, has fast growth in cheap cultivating medium, easy manipulation and. with its size in average of 5 – 10 μm it is visible under normal light microscopy [14]. Furthermore, with a very strong membrane the yeast cells can stand for a wide variation of salt concentrations.

Heparin and haemoglobin were selected as inhibitor models in the study since both are natural components of the blood. A number of publications have shown that haemoglobin has a great inhibition effect on PCR and similar effects have been reported with heparin [3, 15, 16]. Haemoglobin is known as a multi-chained protein that is the oxygen-carrying protein of red blood. The haemoglobin is made up of four polypeptides or globin chains; two identical α-chains and other two identical β-chains. The globin chains of the hemoglobin are interacted and connected with each other by the haeme group that consists of an iron ion (Fe^{2+}) in the centre. The haeme group with iron ion has been shown to be involved in inactivating several DNA polymerases in PCR reactions [17] In an initial experiment we determined that a concentration of 1 mg/ml haemoglobin or of 0.013 mg/ml heparin had a great inhibitive effect on our PCR amplifications. A concentration 10.mg/ml of haemoglobin in water and 1.3 mg/ml of heparin in water were therefore selected as suitable inhibitor concentrations for all the tests throughout the study. These concentrations were 1 and 2 orders of magnitude, respectively, higher than the concentration giving PCR inhibition effect.

Several factors define the amount of cells that can be collected by the DEP chip such as the designed geometrical electrode, the conductivity of the sample, the flow-rate, and the field-strength. The conductivity of the sample is an essential factor that influences the effective of the DEP chip. Adding ions to water will increase the conductivity of the solution, and almost any inhibitor will therefore increase the conductivity of the mixture in comparison to clean water. As one can see from equation 3, there is a cross-over point, where positive DEP ceases to exist and the electrodes will not attract the cells. The force by which cells are attracted to the electrodes is reduced as σ_f gets closer to σ_c. The transition from useable to non-useable conductivity to apply for collecting cells is not abrupt, but graduated, as the layer of cells attached to the electrodes becomes thinner as σ_f increases. A high cell collection efficiency of the DEP chip of more than 90% was observed when the conductivity of the fluid was lower than 50 mS/m. Experimental date

revealed that conductivity of 70 mS/m is applicable in our DEP chip using the yeast cell. We also found that, applying a sample with lower conductivity will ensure a larger DEP force and higher flow velocities can be applied for the DEP chip. When the electrodes were saturated, no further cells were collected and every new cell entering the DEP chip would be wasted. We determined that with a yeast culture of 10^7 cells/ml and an electrode surface of 0.8 mm^2, 1 minute was sufficient time to saturate the electrodes.

A number of advantages of the DEP chip used as selected filter are: 1) The cells needed for analysis are attached on the electrodes, and the application of purified water, with lower conductivity, for washing, ensures a positive DEP (the cells are attracted to the electrodes). 2) The cells can easily be released after washing by switching off the electric field. 3) The released cells are ready for analysis in the next step and 4) the chip is ready for another sample analysis. The DEP chip also has a number of disadvantages. A main concern is the limit of suitable conductivity of the fluid carrying the cells. In contrast to stabilization of pH, which can be achieved by a pH-stabilizing buffer, the only way to reduce the conductivity of a solution is to dilute with a solution having a lower conductivity. In our system with yeast cells we used pure water for dilution and washing. However, not all cells can stand for solutions with such low osmotic pressure. In this case a sugar solution with physiological osmolarity (e.g. 9% sucrose) can be used.

With the DEP chip, it is possible to sort different types of cells on the basic of differences of their dielectric properties. Such a method requires a fluid with known conductivity, and the specific DEP spectrum of each type of cell. The DEP spectrum of a certain cell type is in most cases unknown and, when so, laborious to obtain. However, knowing these, it is possible to adjust the frequency and conductivity needed, making the DEP chip capable of discriminating between different cell types e.g. cancer and normal cells [18].

In this study we used pure water for washing sample and the fluid conductivity was therefore lowered during the wash. It is however, possible to use a fluid with a specific conductivity in the wash to keep σ_f constant when discriminating based on cell type.

The DEP chip described in this study was designed with only the electrodes used for collecting and holding the cells. Therefore, it is necessary to release the cells from the electrodes and collect them outside for the PCR analysis. Research on a newly designed integrated chip, in which a PCR amplification chamber is integrated with the DEP part on the chip, is progressing. The newly designed integrated DEP/PCR-chip makes it possibly to move the pretreatment of cells

on chip, this will reduce the loss of rare cells as well as minimize the amount of external handling before a diagnostic PCR analysis.

In conclusion, in the present study we showed that yeast could be selectively withheld while PCR inhibitors are removed by using dielectrophoresis in a microsystem. The required volumes (sample and wash) are small, and in the order of 30 µl. The dielectrophoretic filter can be used to collect any cell type, without altering the set up much. In contrast to conventional methods the sample preparation method presented here is suited for integration with microstructures for PCR reaction and DNA analysis. This work is an important contribution towards sample preparation, PCR reaction and DNA analysis in a micro total analysis system for molecular diagnostics.

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Figure 1. Schematic outline of the DEP microsystem used in this study. The electrode structure is of the interdigitated finger geometry [6].

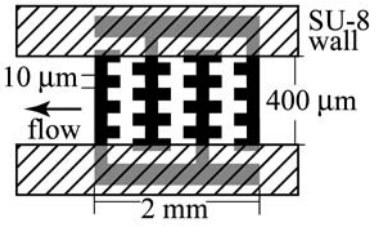


Figure 2. Schematic overview of the set up, showing the first step in which the sample is sucked into the structure.

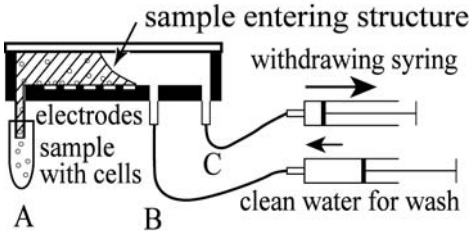
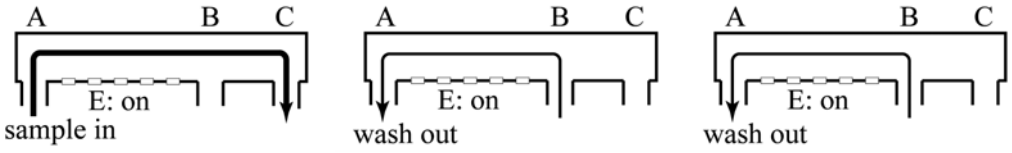


Figure 3. Functional overview of the experiment set up. Step 1, the pump withdraw and the sample is sucked over the electrodes. Step 2, the inhibitor is washed away while the cells are attached to the electrodes. Step 3, the volts applied to the electrodes is turned off. The cells are released and collected outside.



Step 1: Collection Step 2: Washing Step 3: Releasing

Table 1. The inhibition effects of haemoglobin and heparin on PCR.

Haemoglobin	10 mg/ml	1 mg/ml	100 µg/ml	10 µg/ml	1 µg/ml	100 ng/ml	pos	neg
PCR result	–	–	+	+	+	+	+	–
Heparin	1.3 mg/ml	130 µg/ml	13 µg/ml	1.3 µg/ml	130 ng/ml	13 ng/ml	pos	neg
PCR result	–	–	–	+	+	+	+	–

Note: (+):PCR positive

(–): No PCR product.

Pos/neg: positive and negative controls.

Table 2. Removal of haemoglobin and heparin inhibition effects on PCR. The bakers yeast cells were mixed with PCR inhibitors - haemoglobin or heparin, respectively. The cell mixture was applied to the DEP chip to wash away the inhibitors with a flow rate of 0.2 ml/h (see text). The cells were collected at different time points and PCR was performed with the yeast ribosomal protein S3 primers (see Materials and Methods for more details).

Washing time (minutes)	Haemoglobin (10 mg/ml)	Haemoglobin (10 mg/ml)	Heparin (1.3 mg/ml)	Heparin (1.3 mg/ml)
0	–	–	–	–
2.5	–	–	–	–
5	+	+	–	–
7.5	nt.	+	nt.	–
10	+	+	+	+
30	+	nt.	+	nt.
Positive control	+	+	+	+
Negative control	–	–	–	–
Inhibitor control	–	–	–	–

Note: '+': Indicates a strong peak on the electropherogram.
'–': Indicates no peak.
'nt.': Not tested.

7 Discussion and Conclusion

The primary goal of this thesis has been to establish two different biological assays in microchips: micro-flow cytometry and DNA amplification by PCR with embedded heaters. Both goals have successfully been achieved in this work. Furthermore, sample pretreatment by DEP for PCR has been presented.

Two different chip designs for micro flow cytometry have been demonstrated and discussed in Chapter 2. The first presented chip design is a cross chip. This design is used in a static assay for the application of cytotoxicity. This assay yielded high quality data with low variability, and thereby proved the usefulness of the chip format. The second chip design is a spiral chip, where the application is a dynamic cytotoxicity assay. In this design, the mixing of labeled cells and the detergent Triton X-100 is performed on-chip. This feature gives the advantage of exact determination of assay start time and more uniform reaction conditions. Furthermore, due to the long detection channel of the spiral chip, it is possible to monitor a reaction at multiple time points. The benefits of this chip were used to follow the action of Triton X-100 on cell membranes at three separate observation points. The results from this assay indicate that the effect of Triton X-100 on cell membranes sets in between 0.2 and 25 seconds, and has not yet reached a steady state after 47 seconds.

The topic emphasized in this thesis has been DNA amplification applied on a PCR chip with integrated heaters and temperature sensor. However, before DNA amplification on-chip is possible, it is necessary to passivate the chip surface in order to avoid unspecific attachment of template DNA and *Taq* polymerase. Coating of the chip surface was effectively accomplished with silanization by vapor deposition of dichlorodimethylsilane. This treatment proved successful in decreasing the amount of template DNA needed for amplification. PCR on-chip was only possible if extra *Taq* polymerase was added to PCR solution. However, due to the low volume of PCR solution in amplifications performed on-chip, the *Taq* polymerase consumption is not increased considerably.

Characterization supported by simulations have shown that the PCR chip with integrated heaters and temperature sensor have excellent thermocycling capabilities. Maximum heating and cooling

rates of 50°C/s and 30°C/s respectively have been obtained. This means total transition times only account for as little as 5 seconds in a PCR cycle. This PCR chip is therefore able to perform rapid amplifications due to the fast PCR thermocycling.

The practical use for the PCR chip was demonstrated by *Campylobacter* detection from samples of various sources (Chapter 5). The PCR chip proved successful in amplification of DNA from all tested samples. It was even possible to perform PCR using whole *Campylobacter jejuni* cells as template. On average the PCR yield from chip was 68% compared to standard tubes. At several occasions the outcome was almost the same as in tubes, and a PCR yield of 131% was also acquired. The detection time has decreased significantly with the chip detection method, compared to conventional detection methods. This chip method can be performed in less than a day, where a couple of days is needed for detection using the conventional method.

Finally, an example of sample pretreatment that can be integrated with PCR is demonstrated. In this pretreatment method cells are captured by DEP force, which is a highly selective technique dependent on the cellular composition. In the presented example yeast cells were separated from the known PCR inhibitors haemoglobin or heparin. Subsequent PCR was successfully performed on the separated whole yeast cells. The separation by DEP in a microsystem is thereby demonstrated as an effective sample pretreatment method.

In summary, the main goals have both been accomplished as flow cytometry and DNA amplification are established in microformat. For both achievements the resulting device has a novel approach. Whether it is on the application itself or the material it is made of, both approaches imply that the perspectives of the two devices have expanded. Further development and research in the presented devices will most likely lead to new applications and biological assays, as both devices have large potentials in the field of biological and biochemical analytical sciences.

8 Outlook

With the successful demonstrations of devices for flow cytometry, PCR, and sample pretreatment, this thesis has contributed to the ever increasing 'tool box' of microfabricated structures. The multiple functionalities in the microfabricated 'tool box' will hopefully appear in some years as part of new integrated devices, or perhaps in new 'point-of-care' apparatus at the general practitioner.

8.1 Flow cytometry

With the demonstrated on-chip diffusional mixing, it is possible to follow a reaction from the very start and on-wards (Chapter 2). Therefore, it is possible to measure fast reacting reagents. Measurements can be done at any point along the detection channel, depending on the reaction kinetics. The spiral chip therefore broadens the use of micro flow cytometry.

With the long detection channel illustrated in the spiral structure (Chapter 2), it is possible to follow a complete reaction from start to end. By placing the optics at appropriate points down the detection channel, the kinetic of a reaction can be revealed in a single experiment. Only three detection points were used in the demonstrated dynamic cytotoxicity assay. This number of detection points can only give an indication of how the kinetics are in an investigated reaction. However, in future use of the spiral chip, more detection points should be used for studying reaction kinetics, as more detection points gives a better resolution.

8.2 PCR chip

Successful, fast amplification was achieved in the PCR chip. However, the PCR chip can presumably perform even better. For historical developmental reasons the volume of the PCR chip have not changed from 20 μl since the early design. Nevertheless, the volume of the PCR chip can be decreased to 5 μl, with a simple change in the thickness of the SU-8 wall. With the lower volume, an even faster amplification can be performed on the PCR chip. However, as the surface to volume ratio will increase in the 5 μl PCR chamber, this possibility is only suitable if silanization is optimal.

Before it was possible to perform amplification on the SU-8 surface, it was necessary to silanize it. Further investigations should be performed to improve the silanization technique. With optimal silanization the PCR efficiency on-chip will be equivalent, or better than, compared to tubes.

Even that it is necessary to treat the SU-8 surface in the PCR chamber, the usefulness of SU-8 is in some cases superior to other materials. SU-8 is for example an excellent material as regards to integration of different devices into a single microstructure. Such an integrated SU-8 microstructure is already realized in a device combining sample pretreatment and PCR (Section 1.3.2, (El-Ali, Perch-Nielsen et al. 2003; Perch-Nielsen 2003)). Also integration of waveguides have been achieved in a SU-8 device (El-Ali 2003).

The integrated waveguides opens up the possibility for a microfabricated real-time PCR device. In the mixture for real-time PCR, a dye or a probe is included to give a fluorescent signal of the DNA in solution. The real-time PCR device will use waveguides for exiting the dye or probe, and also have the possibility to transmit emitted fluorescent light to a detector. In the real-time device the separate detection step normally performed off chip is eliminated, as this step is performed in real-time as PCR is executed. In addition to the fast PCR the eliminated detection step will diminish the overall analysis time for PCR performed on-chip.

8.3 Sample pretreatment

In the sample pretreatment example illustrated in Chapter 6, the subsequent PCR is performed off chip in conventional PCR apparatus. However, as mentioned above, sample pretreatment and PCR has been integrated. Although this device has not been tested thoroughly, the initial testing looks promising. With this integrated device, the time necessary for detection of, for example, a genetic character from cells in a raw biological sample can be diminished considerable. The dimished detection time and low reagent consumption are features that open up the possibility for this integrated device to develop into a ‘point-of-care’ apparatus – the ultimate goal for a μTAS.

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