Microchips for Isothermal Amplification of RNA

- Development of microsystems for analysis of bacteria, virii and cells

Thesis submitted for the degree of Doctor scientiarum

by

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Anja Gulliksen

Summary

The goal of the present work was to develop a microchip for amplification and detection of mRNA by employing nucleic acid sequence-based amplification (NASBA) technology. The technology platform should in principle be adaptable for any clinical analysis using mRNA or ssDNA as a target. To demonstrate the microchip functionality, identification of human papillomavirus (HPV) type 16, the etiological agent for cervical cancer has been used.

The work shows for the first time successful real-time amplification and detection employing NASBA in microsystem formats using custom-made instruments. The first silicon-glass chips contained reaction chambers of 10 nl and 50 nl, which decreased the NASBA reaction volume by a factor of 2000 and 400, respectively. Further, experiments employing cyclic olefin copolymer (COC) microchips for simultaneous amplification and detection, automatically distributed the sample into 10 parallel reaction channels with detection volumes of 80 nl. In order to detect the simultaneous amplification in the reaction channels, a second custom-made optical detection system with increased sensitivity, heat regulation and an automatic non-contact pumping mechanism, was made. Dilution series of both artificial HPV 16 oligonucleotides and SiHa cell lines showed that the detection limits for the microchips were comparable to those obtained for experiments performed in conventional routine-based laboratory-systems. For experiments related to the development of a self-contained microchip for NASBA, the detection volume was increased to 500 nl due to the advantage of an increased fluorescence signal.

For the NASBA reaction, biocompatible surfaces are critical. It was not possible to amplify any target in microchips with native silicon or COC surfaces. Adsorption measurements indicated clearly that fluorescently labelled mouse IgG bound non-specifically to the hydrophobic native COC surfaces, while PEG coated COC surfaces showed adequate protein resistance. Of the coatings tested for the COC microchips, surfaces modified with PEG showed the best biocompatibility. Successful amplification was obtained with silicon microchips when the surfaces were modified with either SigmaCote™ or SiO_2.

In order to integrate the NASBA reagents on chip, a thorough evaluation of the reagents to be spotted and dried was performed. Because of the limited number of microchips

available, it was necessary to map the most critical parameters on macroscale prior to transfer to the microscale. The DMSO and sorbitol enclosed in the standard NASBA reaction mixture were difficult to dry, and therefore it was necessary to add these compounds to the oligonucleotides or the sample of extracted nucleic acids before the sample was applied on the amplification chip. The standard NASBA reagents consist of the two main solutions, mastermix and enzymes, in addition to the sample. Both the mastermix and the enzymes were stable only when spotted and dried separately. Protectants, such as PEG and trehalose were essential for recovery of enzymatic activity after drying on macroscale. The times for diffusion of modified molecular beacons in dried mastermix and of fluorescently labelled mouse IgG in the dried enzyme solution were ~60 seconds and ~10 minutes, respectively. So far, only dried enzymes with 0.05% PEG protectant have been successfully amplified on chip. Successful amplification using a rehydrated mastermix on microchip still remains.

Optimal design and fabrication methods of the microchips were found to be crucial for chip performance. Rough surfaces do not only create background noise for the optical measurements, but it also contributes to generation of bubbles and problems related to manipulation of the sample within the channel network. The silicon microchips were manufactured with optically smooth surfaces. However, low surface roughness was not easily obtained for the COC microchips. Of the fabrication methods evaluated, it was the injection moulded chips which showed the smoothest surfaces, closely followed by the hot embossed chips. Milled and laser ablated chips produced the roughest surfaces.

A novel non-contact pumping mechanism based on on-chip flexible COC membranes, combined with actuation pins in the surrounding instrument, was tested and evaluated. The mechanism enabled metering, isolation and movement of nanoliter sized sample plugs in parallel reaction channels. The COC chips with integrated pumps were able to simultaneously move parallel sample plugs along the reaction channels in four different positions. Each reaction channel contained a set of 4 actuation chambers in order to obtain metering, isolation and movement of the sample plug into the detection area. The pump accuracy depended on the evaporation of sample and the deformation of the COC membranes.

The results presented in this work are promising with regard to the development of a complete integrated and self-contained mRNA amplification microchip for multi-parallel target testing of clinical samples.

List of Abbreviations

Abbreviation	Explanation
µTAS	micro total analysis system
AMV-RT	avian myeloblastosis virus reverse transcriptase
Bp	base par
BSA	bovine serum albumin
cDNA	complementary DNA
CLSM	confocal laser scanning microscopy
CNC	computer numerical control
COC	cyclic olefin copolymer
dabcyl	4-(4'-dimethylaminophenylazo) benzoic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DRIE	deep reactive ion etching
dsDNA	double-stranded DNA
ECL	electrochemiluminescence
ELISA	enzyme-linked immunosorbent assay
ELGA	enzyme-linked gel assay
FAM	6-carboxy-fluorescein
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
HPV	human papillomavirus
LCR	ligase chain reaction
LED	light emitting diode
LOC	lab-on-a-chip
LOD	limit of detection
mRNA	messenger RNA
NASBA	nucleic sequence-based amplification
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEO	polyethylene oxide
PMT	photomultiplier tube
POC	point-of-care
POE	polyoxyethylene
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RNase H	ribonuclease H
rNTP	ribonucleoside triphosphates

rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction
siRNA	small interfering RNA
ssDNA	single-stranded DNA
T_g	the glass transition temperature
tRNA	transfer RNA
TTP	time-to-positivity

List of Publications

Papers included in the thesis

I. Anja Gulliksen, Lars Solli, Frank Karlsen, Henrik Rogne, Eivind Hovig, Trine Nordstrøm and Reidun Sirevåg, **Real-time Nucleic Acid Sequence-Based Amplification in Nanoliter Volumes**, *Analytical Chemistry*, 2004, 76, 9 – 14

II. Anja Gulliksen, Lars Solli, Klaus Stefan Drese, Olaf Sörensen, Frank Karlsen, Henrik Rogne, Eivind Hovig and Reidun Sirevåg, **Parallel Nanoliter Detection of Cancer Markers using Polymer Microchips**, *Lab on a Chip*, 2005, 5, 416 – 420

III. Lars A. Solli, Anja Gulliksen, Olaf Sörensen, Frank Karlsen, Lars R. Sætran, Liv Furuberg, Henrik Rogne and Klaus S. Drese, **A novel non-contact pump mechanism for parallel movement of nanoliter sized liquid plugs using flexible diaphragms**, *manuscript*

IV. Anja Gulliksen, Michal Mielnik, Bente F. Hoaas, Eivind Hovig, Frank Karlsen, Henrik Rogne and Reidun Sirevåg, **Aspects towards the development of an isothermal amplification microchip**, *manuscript*

Introduction

Since the mid-1980s, researchers have repeatedly demonstrated the practical use of nucleic acid based assays for clinical laboratory work. Since then, nucleic acid based technologies have advanced rapidly, resulting in improved sensitivity, specificity, speed and ease of use. Potential applications for nucleic acid technologies are broad, including detection of pathogens in clinical medicine, genetic screening and diagnosis, monitoring therapy and persistence of infection during drug treatment. In addition, it has also been applied to veterinary medicine, food safety, and forensic analysis.

The identification of unique DNA or RNA sequences or differentially regulated specific genes in an individual, may indicate the presence of genetic diseases or conditions, such as precancerous states or predisposition to cancer itself, tissue compatibility, or bacterial and/or viral infections. Since messenger RNAs (mRNA) are directly involved in all biological activities in eukaryotic cells, bacteria and virii, mRNA can give valuable and important clinical diagnostic information. For this reason, mRNA, rather than DNA, is often preferred as a clinical diagnostic marker.

However, in many cases, target nucleic acids may be present only in very small quantities, making it difficult or impossible to detect by direct analysis. In such cases, nucleic acid amplification is often employed in order to increase the number of copies of the target to a detectable level. For detecting mRNA, two of the most commonly used amplification techniques are reverse transcriptase polymerase chain reaction (RT-PCR) and nucleic acid sequence-based amplification (NASBA).[1, 2]

A few years back, nucleic acid assay based analyses were mainly utilized as research tools, rather than for routine diagnostic surveillance. However, in recent years nucleic acid assays have become more commonly used for diagnostics.[2, 3, 4] Today almost all clinical samples of tissue, blood etc., taken from patients at the local doctor's office, are sent to remote laboratories for analysis. This is expensive and time consuming, increases patient anxiety, and it delays the start of possible treatment. Ideally, such analyses ought to be performed at the local doctor's office by using fully automatic and accurate micro total analysis systems (μTAS), a technology whose development was initiated in the early 1990's. The concept of

μTAS builds on performing all the necessary steps that are required for a chemical analysis, such as, sample preparation, chemical reactions, analyte separation, analyte purification, analyte detection, and data analysis, in an integrated and automated fashion on a miniaturized device.[5, 6, 7] The chemical analyses are preferably performed on inexpensive disposable microchips in order to cut down on costs of production and to avoid contamination issues. Another term widely used is lab-on-a-chip (LOC), which indicates generally the scaling down of single or multiple lab processes to a chip format. The goal is to achieve increased efficiency through smaller scales, and to undertake analysis that cannot be done conveniently by other means. Typical channel diameters are, usually, between, ten to several hundred micrometers, see Figure 1. Microfluidics deals with the behavior, precise control and manipulation of small volumes of fluids. The field of microfluidics has made it possible to facilitate with high accuracy, the handling of sample and reagents of extremely small volumes (smaller then a few picoliters).[8]

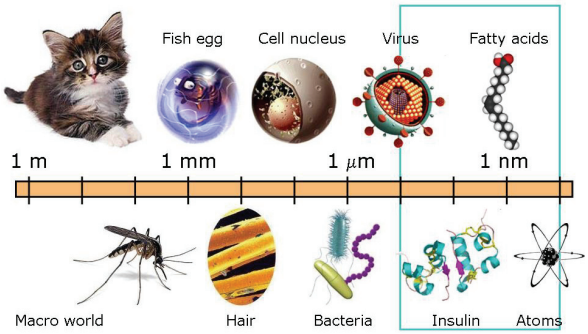

Figure 1 A perspective of the certain objects found in every day life related to size. The order of magnitude defined for nanotechnology lies within the blue rectangle. (The image is adapted form the Norwegian Research Council.)

There are several obvious advantages related to the miniaturization of biological assays.[9, 10] In general, key advantages of these microfabricated systems are:

- Complete automation (sample in – answer out)
- Reduced sample and reagent consumption, resulting in less waste
- Higher analysis control and efficiency due to short mixing times

- Better process control, because of a faster response of the system
- Increased sensitivity and specificity
- Online and real-time monitoring
- High throughput due to reduced analysis times and parallel sample processing
- Reduce the risk for carry-over contamination because of disposable chips
- Reduced exposure to hazardous samples and procedures
- Cost-effective disposable chips because of mass production
- Lower power requirements enable portable systems which can be used in the field
- Lower cost of analysis due to reduced amounts of expensive reagents
- Automatization of molecular assays eliminate manual handling standardizing the protocol which avoid variability between laboratories and within a laboratory

Clinical molecular diagnostics is predicted to be one of the most promising applications for these miniaturized LOC systems, in particular with respect to point-of-care (POC) testing.[11, 12, 13, 14, 15, 16, 17]

Scope and outline of this thesis

The purpose of this work has been to develop a microchip for amplification of mRNA, which can be used for diagnosis. The NASBA technology was chosen as the amplification method because it was well documented for analysis of clinical samples.[18, 19, 20, 21] This method has the advantage of being isothermal, thus avoiding the need for thermocycling at high temperatures, which is necessary in the case of a RT-PCR approach.

A microchip for amplification and detection of RNA can function both as a single unit chip, but also as a part of a larger integrated diagnostic POC instrument. A complete automatic diagnostic instrument would need a sample preparation unit which can receive and treat fresh clinical samples to obtain a pure solution of nucleic acids, which in turn, can be transferred to an amplification chip.

The work to develop a diagnostic microchip for mRNA analysis was approached in the following manner:

1. Downscaling of the NASBA reaction to the nanoliter level using silicon-glass chips and a custom-made optical system for fluorescent detection.
2. Development of a cyclic olefin copolymer (COC) chip in order to distribute one sample into parallel reaction channels for simultaneous parallel amplification of mRNA. The limit of detection (LOD) of cell lines and synthetic oligonucleotides were examined on microchips and compared to that observed in conventional systems.
3. Integration of NASBA reagents on the microchips was performed by deposition and drying in specific areas, to obtain fully automatic diagnostic systems.

In the following chapters a detailed description of the various techniques used in this work is presented. Chapter 1 briefly summarizes the transcription-based amplification technology and discusses the differences between RNA and DNA, and the significance of concentration in a clinical setting. An introduction to the μTAS technology is found in Chapter 2. Then follows short overviews of microchips currently on the market, choice of material, fabrication technologies, surface modification, storage of reagents, actuation, and detection mechanisms. Chapter 3 gives a summary of the 4 appended papers. In Chapter 4, the results obtained in this work are discussed. Concluding remarks and future perspectives are found in Chapter 5.

1 Background

Cancer affects more people than any other disease. About one third of the world population is likely to get this diagnosis during their lifetime. Human papillomavirus (HPV) is considered the etiological agent for cervical cancer, which is the second most common female cancer form world wide and the third most common cancer regarding mortality.[22, 23, 24] The activity of this virus has the potential to start the production of harmful proteins, which might stimulate growth of cervical cells which will, eventually, lead to the loss of cell cycle control. Cervical cancer is currently diagnosed by cytological methods, which have poor reproducibility and limited sensitivity.[25] A molecular based diagnostic LOC system, placed at the local doctor's office, would quickly identify multiple high-risk HPV mRNA transcripts of all women with persistent transforming infection with higher accuracy and reproducibility in comparison with conventional cytology. This work has focused on making microchips for detection of HPV which is the core knowledge of NorChip AS. However, the microchips are general detection platforms and the target to be analysed on the chip can easily be changed.[26]

1.1 RNA versus DNA

DNA molecules contain the coding sequences for RNA and protein molecules, of which the latter are the molecules actually performing the work in a biological system. Figure 2 shows the sequence of events from the genomic DNA in the nucleus of a eukaryotic cell to the synthesis of proteins in the cytoplasma.

RNA has the same coding sequence as DNA, but in contrast to DNA, RNA is either directly or indirectly involved in the processes of the machinery of a cell. Different RNA (*e.g.* tRNA, rRNA, siRNA, RNAi) molecules form three-dimensional structures that are directly involved in the regulation or activation of the biological processes in the cell. In contrast to DNA, mRNA, therefore, provides important information about the various activities of eukaryotic cells, bacteria and virii. Since ribosomal RNA (rRNA) can persist for long periods in dead cells, attention has turned to the use of mRNA as a marker for viability. Messenger RNA usually has a short half-life (seconds) within viable cells, due to rapid degradation by

specific enzymes (RNases) which are themselves very stable even in environments outside the cell itself.[2, 27] However, mRNA degradation can be dependent on the susceptibility of the transcript, or regions thereof. Synthesis of mRNA in viable cells may depend on the physiological state of the cells, so the targeted mRNA transcripts of genes should be constitutively expressed in all physiological states. By choosing RNA instead of DNA a higher number of molecules will be available to serve as targets. Even in cells with low levels of expression, the number of RNA copies will exceed the number of DNA copies. An exception to this rule is cells with integrated virus which is not transcribed and can only be detected after DNA amplification or by direct hybridization.

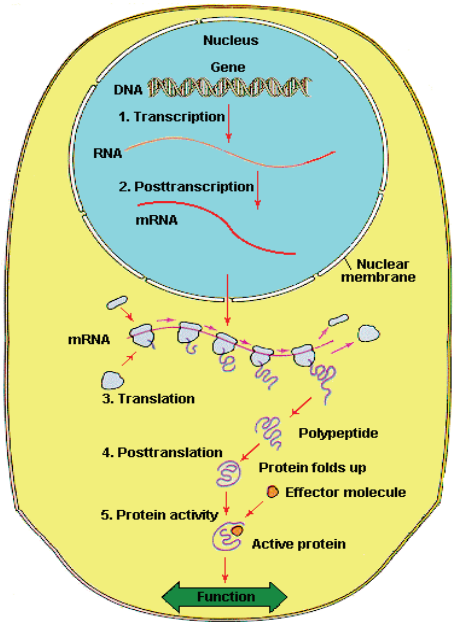

Figure 2 A schematic diagram of the fundamental processes from DNA to protein in a eukaryotic cell. DNA undergoes a transcription resulting in mRNA in the nucleus. The intermediated mRNA will again be translated to proteins by assembling amino acids resulting in polypeptide chains with the help of ribosomes and tRNA in the cells cytoplasma.

Proteins would give the most accurate clinical description of viability of the cells, but the use of a protein as target for routine diagnostics has the disadvantage of low sensitivity, reproducibility and specificity. In contrast, the main disadvantage using DNA as a target for routine diagnostics has been the lack of information about biological or clinical activity. During the last decades, microarray technology and varied amplification methods have shown that mRNA is a valid target for routine molecular diagnostics and for future POC testing.[11, 28]

Using mRNA as a target for routine diagnostics may provide information of clinical activity, regulation or processes, in addition to higher or equal sensitivity, reproducibility and specificity to DNA.

1.2 NASBA

NASBA is a transcription-based method which can amplify any RNA and single-stranded DNA (ssDNA) sequence isothermally (41°C), by the simultaneous use of the activities of the three enzymes; avian myeloblastosis virus reverse transcriptase (AMV-RT), ribonuclease H (RNase H) and T7 RNA polymerase.[29] A schematic diagram of the NASBA reaction is shown in Figure 3. Target sensitivity and specificity are dependent on the efficiency of the hybridization kinetics of the two primers, the molecular beacon probes, the three-dimensional polymer structures surrounding the target, and the quality of the target.[30, 31] However as a rule, more than $10^9 – 10^{14}$ antisense RNA molecules can be generated in about 1.5 hours.[29, 32, 33]

NASBA is highly specific for RNA and that only in the absence of target RNA or in case of an large excess (> 1000-fold) of DNA over RNA, can DNA be amplified in NASBA.[34, 35] The sensitivity of the assay decreases drastically when DNA is used as target as compared with the corresponding RNA. This indicates that even in the presence of identical amounts of RNA and DNA, the RNA will out-compete the DNA for the enzymes used in NASBA, due to the higher affinity for RNA. However, in general the reaction is not affected by double-stranded DNA (dsDNA) contamination.

Today, one of the most commonly used methods for mRNA detection is RT-PCR. This method makes first use of reverse transcriptase to produce RNA:DNA hybrids, which then is followed by a PCR amplification. The main product of this reaction is dsDNA. Using PCR, the number of molecules doubles for each step, and thus it requires approximately 20 cycles to amplify one million-fold.[29] With NASBA, however, 10 – 100 copies of RNA are generated in each transcription step, so fewer amplification steps are required to achieve a similar amplification rate. Consequently, both the total incubation time and the overall error frequencies are reduced with NASBA. Errors that are inherent in some enzymatic reactions (for example, reverse transcriptase) are cumulative, and therefore one would expect that fewer cycles reduce such errors. Consistently, samples with as few as ten molecules of input produce

positive results, and samples with even fewer input molecules (as determined statistically) are detectable. Thus, intron-flanking primers or DNase treatment is not required when mRNA or retroviral RNA is to be analyzed with NASBA, which in contrast is necessary for *e.g.* RT-PCR. DNase treatment, by itself, is not completely effective for all purposes, and the use of intron-flanking primers to distinguish between mRNA- and DNA-derived amplicons[36] is not suitable for prokaryotes.[37] In contrast to RT-PCR, NASBA is isothermal and does not require thermocycling. Microchips along with detection instruments can therefore be made less complex than for RT-PCR.

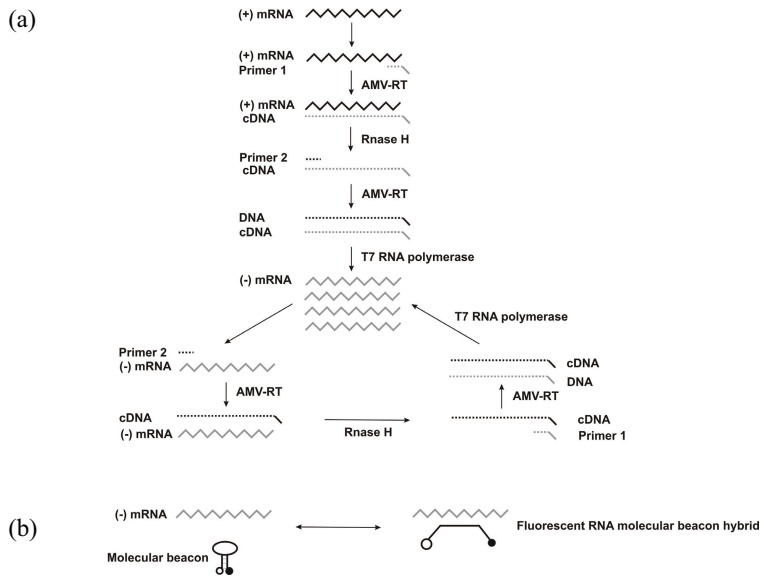

Figure 3 (a) Schematic diagram of the principle of NASBA. Due to the activity of AMV-RT, primer 1 (with a 5'-terminal T7 promoter sequence) is extended, resulting in a RNA:cDNA hybrid. Simultaneously, RNase H degrades parts of the RNA of the hybrid, which in turn makes possible annealing of the target specific primer 2 to the newly formed cDNA. Primer 2 is extended by AMV-RT, resulting in a double-stranded DNA (dsDNA) molecule with a functional T7 promoter. The T7 RNA polymerase recognizes and binds to the T7 promoter sequence and generate RNA transcripts complementary to the original RNA sequence.[38, 39, 40] The newly formed antisense RNA molecules are templates for the synthesis of dsDNA molecules, which again can be transcribed. At this step, the amplification process starts with primer 2, because the newly generated RNA template is antisense to the original target. (b) The amplification reaction is possible to monitor in real-time, due to the fluorescent light produced by the molecular beacon probes when they hybridize to the amplified antisense RNA.

1.3 Limit of detection

Reduction of the volumes in microsystems will decrease the absolute number of molecules available for detection. Hence, the microfluidic systems' ability to manipulate small volumes of fluid is one of the strengths, but also a weakness, because low numbers of molecules are more difficult to detect.[41] However, small volumes are not always suitable for diagnostic applications, due to the limited number of target molecules in the sample fluid. In Figure 4, a plot of sample volume versus analyte concentration (copy number) is shown, which indicates the minimum volume required for statistically significant detection of analyte.[42]

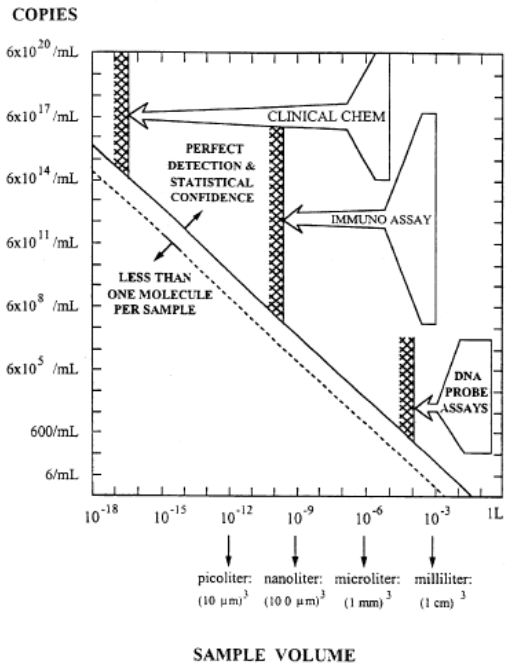

Figure 4 The minimum volume required for statistically significant detection of analyte. The plot shows sample volume versus analyte concentration (copy number).[42]

In the case of infectious diseases, bacteria may be present at less than 10 copies per milliliter of blood. For drinking water and food, only a few bacteria per liter or gram of food is sufficient to cause disease.[42] Therefore, the very nature of microfluidics devices makes sample

concentration a necessary task prior to detection in almost any LOC application to enhance the detection limit. Figure 5 and Table 1 illustrate the relation between fluid volumes, number of molecules within the fluid volume and the physical dimensions.

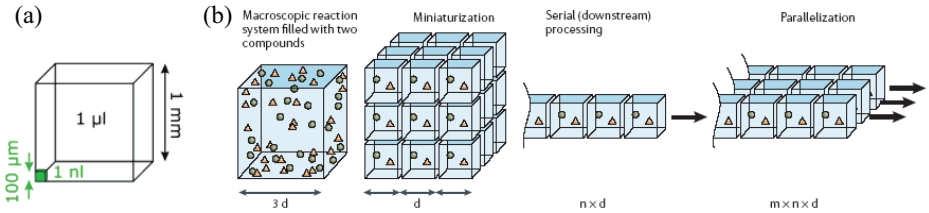

Figure 5 (a) Illustration of the relation between volumes and dimensions. (b) Miniaturization of a macroscopic reaction system.[43] d, length of edge; n and m, numbers of reaction systems serial and parallel, respectively.

Table 1 Volumes with correlating dimensions and number of molecules within the volume.

Volume	**1 µl (10^{-6} l)**	**1 nl (10^{-9} l)**	**1 pl (10^{-12} l)**	**1 fl (10^{-15} l)**
Cube dimension	1 mm	100 µm	10 µm	1 µm
Number of molecules (1 µM solution)	6×10^{11}	6×10^{8}	6×10^{5}	600

The sensitivity of miniaturized chemical analysis systems usually depends on the obtainable detection limit which is mainly determined by capabilities of the detector of the system. Therefore, adequate, high-sensitivity detection techniques are indispensable in this field. In addition, it is important to have in mind that the detection limit of an assay generally is restricted by the background signal, *i.e.* the signal for zero analyte concentration.[44] The two key factors for choosing a detection method for microfluidic devices are sensitivity of the method and its ability to be scaled down.[45]

UV-visible absorbance detection is the most commonly used approach for detection in flow-based chemical analysis, whilst fluorescence detection is more commonly used in conjunction with microdevices.[45] Fluorescence differs in principle from absorbance, in that excitation and fluorescence occur at different wavelengths. In the case of fluorescence, one is no longer dependent on the ratio of the two signals at a single wavelength, but is instead looking for a signal of light at other wavelengths. In order to minimize signal-to-noise ratio the

excitation source and the detector are often placed in an angle, since spectral filtering not always is enough. The small dimensions of microfluidic channels limit the path length available for absorbance measurements, severely diminishing the sensitivity of absorbance measurement on-chip. For fluorescent measurements, the fluorescent signal of a single molecule is independent of the dimensions of the detection volume and remains constant.[46] However, the background signal that is generated by impurities in the sample, stray light, and scattering scales linearly with the size of the detection volume.[47] Optical detection requires an optical geometry consisting of a light source, lenses, filters, diffractive elements and detectors, making the instrument bulky. Consequently, efforts have been made to miniaturize this equipment and integrate micro-optical elements into microfluidic devices associated with optical detection.[45, 48]

Other labelled and label-free optical methods such as chemiluminescence, electroluminescence, absorbance, refractive index detection, radioactivity-based, Raman spectroscopy, surface plasmon resonance and thermooptics, as well as electrochemical methods of amperometry, conductimetry and potentiometry are also being studied within the field of microsystems.[48, 49, 50] Electrochemical detection has the potential to be very compact and fully integrated detection method within microsystems, because the analyte is detected by small and compact electrodes, and detection is dependent on electrode surface area rather than on available detection volume.[51] The limits of detection (in concentration terms) do not degrade as rapidly for electrochemical detection as they would for optical techniques. Electrochemical detection is generally attractive for this reason, however, they are far inferior in comparison to fluorescence in terms of sensitivity.[45] Additionally, mass spectrometric detection method schemes have been tested on-chip.[48] Detection technologies for miniaturization are being pursued by either downscaling existing methods or trying new approaches.[49]

When employing fluorescent molecular beacons in NASBA, rapid, specific and sensitive real-time RNA amplification are accomplished.[30] Other detection technologies would require post-NASBA detection and would increase the complexity of the microchip and instrument. In order to develop the simplest microsystem for the easiest operation, fluorescent detection was chosen.

2 Microchips

2.1 Status of microchips

Future LOC devices may revolutionize the way doctors diagnose disease and monitor treatment. Portable devices for rapid automated analysis of body fluids or tissue samples from sample preparation to data readout could be performed within minutes or even seconds. The electrophoresis chip, LabChip® from Caliper/Agilent is considered the first LOC product on the market (Figure 6a). A portable blood analyzer system, i-STAT, from Abbott Point of care is also available (Figure 6b).[52, 53] Cepheid has also launched the GeneXpert® which consists of a automated sample preparation cartridge and a thermocycler for PCR/RT-PCR with optical fluorescent detection in ~ 35 minutes (Figure 6c).[10, 54, 55] The microfluidics-based cartridge consists of multiple chambers that are designed to hold the biological sample in lysis buffer; purification and elution buffers; PCR/RT-PCR reagents, and to retain all sample-processing waste. Wet reagents[10] or lyophilized PCR reagents in bead form[55] are loaded manually into the cartridge reservoirs before the assay is performed, and all subsequent fluid movement within the cartridge is controlled by the software. The plastic cartridge has a large detection volume (80 µl) and can detect up to four different fluorescent colours.[10] Other companies engaged in the field to explore the potential of microfluidics include *e.g.*: Affymetrix, Agilent Technologies, Alderon Biosciences, Caliper Life sciences, Cepheid, eBiochip Systems, GenProbe, Idaho Technology, Nanogen, Nanosphere, Roche Molecular Diagnostics, HandyLab, Micronics, Gyros, Micronit, Motorola, Texas Instruments, Samsung and Philips.[56] Over the past years several companies have also been established to provide fabrication facilities and off-the-self solutions of microfluidic components.[57, 58, 59]

So far, many assays have been applied on chip, such as sample preparation, genotyping, sequencing, purification, separation, hybridization and amplification of nucleic acids, drug screening, and single cell manipulation. Several other diagnostic systems are being developed as well. Currently, one of the most common research areas of microfluidic devices is in connection with nucleic acid analysis.[11, 48, 60] Northrup *et al*., in 1993, were the first to report on PCR on a microchip.[61] Since then, the implementation of PCR on chip has been accomplished using several different approaches. A thorough description of amplifying DNA

using different PCR approaches on chip can be found in the in the numerous reviews available.[62, 63, 64, 65, 66]

Figure 6 LOC on the market. (a) The electrophoresis chips, LabChip® from Caliper/Agilent.[52] (b) i-STAT blood analyzer from Abbot Point of care.[52] (c) Self-contained cartridges of the GeneXpert® system from Cepheid.

However, only a few groups have described simultaneous amplification and detection of mRNA by RT-PCR on chip.[67, 68, 69,70, 71, 72, 73] In the first studies of amplification of mRNA on chip, the material was removed from the chips for analysis after the reaction had taken place. The reaction volumes were also quite large, in the microliter range.[69, 70, 71] Recently, Marcus *et al*.,[72] on the other hand, have demonstrated a microfluidic chip assay with 72 parallel RT-PCR reactions with 450 pl reaction chambers and endpoint detection. The system was able to detect less then 50 β-actin transcripts from a total RNA template.

Only few reports describing the use of NASBA on microchips have been published. In addition to the work presented in this thesis, only Xtrana Inc. (Broomfield, CO) has presented data and a prototype of a fully automatic microchip-based system performing NASBA on chip.[74] The microfluidic card demonstrates sample preparation, nucleic acid extraction and amplification on solid phase material and lateral flow strip detection. The drawbacks of the Xtrana approach are relatively large reaction volumes (10µl) in addition to only one single target detection and no real-time detection, resulting in time-consuming analyses.

Researchers from Cornell University (Ithaca, NY) and Innovative Biotechnologies International, Inc., (Grand Island, NY) have used macroscale NASBA-amplified RNA sequences to generate material to be used as samples in connection with the development of a microfluidic biosensor for rapid identification of pathogenic organisms and viruses. In these experiments, the microchips do not perform any amplification on the chip. The biosensors have been based on fluorescence detection and bead-based sandwich hybridization and lateral-

flow assays.[75, 76, 77, 78, 79, 80] The methods require incubation with capture probes and magnetic beads extending the time with approximately 15-20 minutes.[77, 79]

2.2 Challenges of microchips

As for many other applied research fields, the expectations with regard to rapid commercial implementation of μTAS and LOC systems have been enormous, however, the expectations have not been fulfilled. Since the μTAS technology is relatively new, many challenges are still ahead and more research is necessary before microfluidics platforms can be used to adapt or replace existing macroscale assays. In general, it takes 10 – 15 years between the first scientific breakthrough until large scale commercial implementation.[58] In order for μTAS to become successful, the technology will require both a broad range of different types of components and subsystems, which need to be integrated into complete and functional systems.[81]

Miniaturization is more than simply scaling down well-known systems as the relative importance of forces and processes changes with scale. As the dimensions decrease, the ratio of surface area to system volume increases, and accordingly the relative importance of the surface forces. A fundamental rule for downscaling is that volume-dependent effects ($length^3$) often are of minor importance compared with surface-dependent effects ($length^2$). This gives rise to various effects, characteristic for the microscale flows. Microsystems feature laminar flow, where the viscous forces dominate inertial forces. Hence, turbulence is often unattainable so mixing only occurs through transport of molecules by diffusion. In addition to the familiar pressure-driven flows, fluid motion can be generated by taking advantages of capillary effects, electrical fields and magnetic fields. Microfluidic flows can be precisely controlled and manipulated to an extent not possible in the macroscopic world. At microscale, the temperature and the surface or interfacial tension of the liquids, the chemical properties of surface materials and the geometrical features of the channel walls have a large impact on microfluidic flow.

Some applications such as capillary electrophoresis greatly benefit from an increased surface area-to-volume ratio, while other applications do not. Phenomena such as adsorption become increasingly difficult to deal with in *e.g.* biological assays. Many analytical techniques

in molecular biology are highly sensitive to minor changes in the chemistry of the reaction and, therefore, pose a significant challenge in process scale-down to the microchip format. This is particularly the case in a multi-component reaction, where the concentrations of several components need to be maintained within a fairly small range. The behaviour of many biochemical bench-top reactions has been found to be quite different in the presence of inorganic reaction vessel materials and higher surface area-to-volume ratios encountered in microstructures than in macroscopic reaction systems. While the walls are usually assumed to play only a minor role in the latter, they play a significant role as a reaction component at smaller size scale. However, adsorption can in some degree be controlled by surface modification.

Evaporation usually poses a significant problem for microfluidic devices, although, evaporation can also be exploited for liquid pumping and sample concentration.[82, 83] In most cases, severe problems arise because of liquid loss. This effect is increased at elevated temperatures. The liquid loss can cause change in reagent concentration, complete disappearance of the reagents/sample or operational malfunctioning. By applying pressure on the system, ensuring tight sealing to avoid leakage and minimize diffusion lengths (dead volumes), less liquid will evaporate.

The main cause for gas bubbles in miniaturized chips is that air might be encapsulated while filling the device with liquid.[84] Encapsulated air bubbles usually alter the performance of the device and have to be avoided to get reproducible data. The filling can be disturbed by pinning of the liquid meniscus by surface roughness or impurities which can contribute to incomplete filling and result in trapped air. Since different fabrication methods produce different surface properties, it is important to consider the fabrication method with regard to surface roughness and chemical composition.

μTAS is a multidisciplinary field requiring knowledge of physics, surface chemistry, biology, instrumentation, fluid mechanics, fabrication technology and computer science. It is a challenge to get researchers to communicate and understand the significance of all disciplines in order to build useful and functional μTAS devices. Many future cases of μTAS will be results from the assembly of a microfabricated chips with conventional, possibly miniaturized, components such as pumps, valves, mixers, light sources, deterctors and electronics. The more elements that are miniaturized and integrated into microfluidic devices, the easier it will be to

develop portable instruments for POC technology.[45, 85, 86] However, complete integration of all elements on chip makes it complex, expensive and more prone to error if robust components are not being deployed. The concept of functional integration in μTAS, and all the accompanying advantages, must therefore be balanced against complexity, cost and feasibility. Another problem to solve is the interconnections and packaging of a final product.[43] For μTAS, this requires fluidic, mechanic, optical, and electronic interconnections. Complex engineering is necessary for efficient delivery of fluids into microfluidic systems, which rely on the creation of high fidelity of macro-to-micro interfaces. Furthermore, μTAS must be packaged so they can be handled safely without damaging the delicate microstructures on the chip.

Yet another obstacle which needs to be dealt with is on-chip reagents storage.[15,81] Long-term stability of reagents is required to ensure robust self-contained POC diagnostic systems. In order to be able to use the μTAS not only at the local doctors' office or at home, but also in the field, the reagents should withstand high storage temperatures as freezers or refrigerators not always are available.[15]

One important problem is that limited technology exist concerning sample preparation on chip.[81, 87] The samples might be dilute or complex (blood, saliva, faeces etc.) which in both cases would require special treatment before analysis. In order to isolate the target, large quantities of sample might be required which causes problems when the devices are small. Thus, the microsystems can easily be clogged due to the amount of large particles within the samples.

A significant challenge arising directly from the adoption of small volume systems is to efficiently detect analyte molecules. Detection is undoubtedly one of the primary issues determining the practicality and application of microfluidic systems.[49] Much attention has been paid on the development of miniaturized and sensitive detection methods. In order to obtain optimal detection conditions, stray light, scattering and autofluorescence etc. needs to be eliminated.

2.3 Fabrication of microchips

2.3.1 Silicon and glass versus polymers

Silicon is a well characterized material. It is unique, as it makes the combination of mechanical and electrical function in single devices possible. This permits the integration of thermal cycling devices (a good heat-dissipating substrate) or detection and control circuitry. However, the use of silicon poses some problems, due to optical opacity, cost, difficulty in component integration, and surface characteristics that are not well suited for biological applications. The use of glass instead of silicon in μTAS application is due to the unique properties of glass, *e.g.* resistance too many harsh chemicals, optically transparency, and its dielectric properties. Other advantages of glass are its hardness, high thermal stability, and relative biocompatibility.[88] It is possible to produce pure silicon chips, glass chips or chip hybrids consisting of both silicon and glass. Silicon- and glass-based microfluidic devices are well suited for chemical applications that require strong solvents, high temperatures, or chemically stable surfaces.[89]

Micromachining of silicon and glass involves the use of wet and dry etching, photolithography, and a variety of other techniques, all of which require the use of cleanroom facilities and equipment. This makes the production cost of these devices quite high, which limits their usage as disposable devices.

Recently, the use of polymers rather than silicon and glass chips has been exploited. Polymer-based microchips have emerged as inexpensive and disposable alternatives. The current trend for biomedical applications strongly points towards use of polymer-based substrates.[8] Many common plastics have been used for fabrication of microfluidic chips with excellent device-to-device reproducibility.[48, 90, 91] Polymers have numerous desirable advantages and characteristics as substrates for microfluidic devices. For example the raw materials and fabrication methods are relatively inexpensive. Methods exist which makes fabrication of polymer microchips quite fast and versatile and applicable to both prototyping and high-throughput production.[62, 63, 82, 88, 92, 93, 94, 95]

A key feature for many polymeric materials is superior biocompatibility (low non-specific binding) when compared to silicon and glassy materials.[92] Most polymers exhibit low surface charge which can be advantageous for several applications.[90] It is possible to obtain

flexibility and variation of the coating of the polymer with regard to chemical resistance against acids and alkalis that is superior to silicon substrates which can easily be etched away. The surface properties of polymers can be readily modified to meet the fluidic and/or biocompatibility requirements of a biochemical analysis system. Most detection methods within the biotechnological field involve optical measurements. The transparency of polymers is therefore a major beneficial feature compared to the non-transparent silicon. However, autofluorescence of certain polymeric materials in different regions of the spectrum might pose problems for optical detection techniques.[96] Polymer chips are disposable therefore cross-contamination can be avoided.

The disadvantage of most polymers is poor chemical resistance to non-aqueous solvents, and their surface chemistry can be difficult to control. Many polymers are hydrophobic and therefore the surfaces have to be treated specifically to avoid adsorption and to obtain capillary filling. Fabrication methods, as well as the polymer itself, along with various surface treatments, can influence the surface charge density and charge location.[90] Some plastics contain a number of additives that have an effect on their processing and shelf life. Such additives include fillers, plasticizers, stabilizers, antioxidants and UV stabilizers, which if leaked into the microfluidic network can inhibit certain biochemical assays.[90] Polymeric materials usually have low dielectric breakdown voltages, and the thermal conductivity of most polymers is much lower then for silicon and glass.

Sealing of the microsystems is critical and leaky channels are a frequent problem. Commonly used bonding procedures for silicon and glass chips are; anodic bonding, thermal fusion bonding and adhesion bonding.[88] Dependent on the method, the wafers can be bonded with or without using any adhesives. When joining the two wafers the surfaces must be ultra clean and flat. Precautions are therefore taken to ensure that there is no surface contamination or particles that could prelude a good bond. The bonding procedures often require high voltages (200 V – 1000 V) and temperatures (180˚C – 1000˚C).[51, 97] As for silicon and glass chips, polymer chips have to be sealed to enclose a complete microsystem. Several methods are available for this purpose. Many polymers are thermally bonded at temperatures above their glass transition temperatures (T_g), the temperature at which the polymer begins to soften. However, elevated temperatures cause destruction of the microstructure elements. Another bonding method uses solvent to wet the bonding surfaces. In solvent welding the surfaces of

both polymer parts are slightly wetted and, thus, loosened using specially tailored solvent mixtures. A drawback of this technology is that all surfaces – also the microcapillary structures are entirely exposed to the solvents used. Adhesive bonding uses an intermediate layer to "glue" the substrates. A number of epoxies, UV-curable epoxies and photoresists can be used for adhesive bonding. Also in this case it is difficult to prevent the glue from flowing into the channels by capillary action. Polymer tapes with a pressure sensitive adhesive layer can be used as well. Laser welding is a localized thermal bonding process, in as much as the interface between polymer chip and lid is briefly melted and then cooled again. This technique requires that the polymer chip absorb the laser energy and that the lid is transparent.

Choice of material depends on the application (*e.g.* biocompatibility and optical smoothness). Plastics are appropriate for the channel structures, glass for optical windows, and silicon for high-level electronic functionality.[98] In addition to the more traditional materials, biomaterial and artificial materials, such as calcium alginate, gelatine, biodegradable thermoplastics, photocurable "liquid" Teflon, silicon elastomers, thermoset polyester, and acrylic copolymer have been tested as well.[99]

In the present work, both silicon-glass chips and cyclic olefin copolymer (COC) chips have been tested. As the goal is to produce disposable microchips, most of the tests are performed on polymer. COC has excellent optical properties, which are advantageous for fluorescence-based biochemical analysis due to low autofluorescence and other bio-optical applications. Their light transmittance extends through the visible spectrum into the near UV. They withstand all common sterilization regimes, including gamma radiation, steam and ethylene oxide. They are highly pure and have excellent water-vapour barrier properties and low moisture absorption.[100] In addition, COC has properties well suited for production such as good material flow, low shrinkage and high glass-transition temperatures. COCs are good electrical insulators, with relatively constant electrical properties over a wide range of temperatures and frequencies. Most metallic films exhibit excellent adhesion to the COC material and it is resistant to aqueous acids and bases, as well as most polar organic chemicals such as acetone, methanol, dimethyl sulphoxide (DMSO), and iso-propyl alcohol.[95] However, COC are disturbed by aliphatic and aromatic hydrocarbons, and should not be exposed to solvents such as hexane and toluene, and certain oils and fats.[100]

The COC surfaces are inert and native COC exhibits a contact angle of ~92˚ with water.[95] However, plasma treatment can be used to modify the surfaces of COC substrate to obtain better biocompatibility characteristics. The desirable combination of mechanical, optical, and chemical properties makes COC currently one of the best commercial candidate materials for the mass production of microfluidic chips, in spite of the fact that it is quite expensive compared to the other polymers.[91]

2.3.2 Microfabrication methods

Micromachining technologies have traditionally been silicon-based, due both to the role of this semiconductor in IC technology and its excellent mechanical properties. Today the manufacturing of microfluidic chips has grown into a field of it own, with constant improvement of chip material and fabrication techniques.[99] Microfabrication makes it possible to reproduce the same carefully designed μTAS several times with the same specifications. As a general rule, the choice of fabrication method is determined by several factors, such as available technologies and equipment, cost, speed, fabrication capabilities (*e.g.* desired feature size and profile), and the preferred material substrate. In the present work, five methods were used. Silicon microchips were fabricated using the deep reactive ion etching (DRIE) process developed at Bosch, while the COC polymer microchips were manufactured by milling, laser ablation, injection moulding, and hot embossing. Table 2 gives an overview of some typical characteristics over the microfabrication methods used in this work with regard to the silicon chips and the COC polymer chips.

The DRIE process produce nearly vertical sidewall features. Depending on the process parameters, only slight scalloping of the sidewall will be generated due to the alteration between etching and passivation. When high-aspect ratio (depth:width > 1) and optical smooth surfaces are desired, DRIE is the method of choice.[51] However, the manufacturing of silicon microstructures for applications in μTAS by the DRIE process is an expensive and not very flexible process, quite large volumes are required for each microchip and thus not suited for rapid prototyping of test-devices. The technique is, however, well adapted for large-volume production of commercial products.

Milling cuts polymer material mechanically and computer numerical control (CNC) controls the position and movement of the cutting tool. This makes the milling process flexible

and it is easy to change the design quickly. With CNC milling it is not possible to achieve very small feature sizes, structures with sizes down to 100 μm are typical.88 Low surface roughness in the nanometer range can be obtained under optimized conditions.101 Normally, surface roughnesses of 2 – 10 μm are obtained, Table 2.

Laser ablation involves the use of a high-powered pulsed laser to remove material from a sheet of thermoplastic. In addition to pulse energy, the depth of the ablated channel is also dependent upon the pulse rate and the absorption characteristics of the substrate. Depending on the laser setup, channels can obtain a Gauss distribution. In other cases the channel bottom can be flat, however, overlapping ablation tracks may result in grooves. Laser ablated areas may also result in melted surfaces with large surface roughness. Generally, laser ablated channels have greater surface roughness than imprinted, hot embossed or injection moulded channels. The degree of roughness is highly dependent on the absorption of the polymer at the exciment wavelength. The smallest feature size attainable strongly depends on the quality of the optical system and the laser wavelength. Laser ablation is therefore advantageous for prototyping purposes due to being a direct technique, not requiring any die.[48, 96]

Laser ablation has the capability to modify the surface of channel walls concurrent with microchannel formation. Many reactive species are formed both at the polymer surface and in the gas phase during the laser ablation process. The incorporation or reaction of these ablation products at the nascent channel walls can result in surface chemical functionality that is significantly different from that in the bulk of the polymer.

Both injection moulding and hot embossing requires a die. The die can be made with CNC metal micromachining, electroplating or silicon micromachining. The production of dies is quite time-consuming, and therefore, dies do not offer a convenient method for changing of designs.[89] Considerable effort is put into the design of molded parts and their die, to ensure that the parts will not be trapped in the die, and that the die can be completely filled during the process. The quality of the replication depends on the quality of the fabricated dies.[48] The die can be used many times depending on its mechanical strength which is dependent on the material used. Both injection moulding and hot embossing can reproduce structures with features as small as a few nanometers.[88] Limitations of injection moulding for microfluidics include resolution and material choices. Injection moulded microchips can be ready in only 1-3 minutes and, thus, suited for large-volume productions.[102] However, making only a few

test chips with this process is quite expensive. On the other hand, hot embossing is fairly straight forward, as well as inexpensive, offering low cost devices, provided there is access to the necessary hydraulic press equipment and a patterned die. An overall cycling time of the hot embossing process is in the order of 5-7 minutes.[102] A wide variety of polymers have been successfully hot embossed. The microchips produced from hot embossing are usually one-layer planar structures.[48]

Table 2 Overview of various microfabrication methods with some of their characteristics. All values are for fabrication of COC chips (provided by IMM, Mainz, Germany).

Method	Material	Feature size	Roughness	Aspect ratio	Rapid prototyping	High-throughput	Large volumes
Deep reactive ion etching[1]	Silicon, glass	~5 µm	< 10 nm	20-30	-	+	+
Injection moulding[2]	Thermoplastics	100-200 µm	0.3 µm with polished die	1-2 for 100-200 µm channels[3], 5 and more for larger channels[3]	-	+	+
Hot embossing[2]	Thermoplastics, elastomeres	~100 µm	0.3 µm with polished die	1-2 for 100-200 µm channels[3], 5 and more for larger channels[3]	+	-	-
Milling	Metal, glass, thermoplastics	~100 µm	typically 5-10 µm, with liquid cooling ~2 µm	for 100 µm mills: 1.5, for 200 µm mills: 2; for 300 µm mills and larger 5 and more	+	-	-
Laser ablation	All solids and liquids	10-20 µm	0.5-0.8 µm for small channels, > 5 µm for larger channels due to pulse rate	1.5-2 for small channels, more for larger channels	+	+	+

[1] – Silicon as the material

[2] – Requires mould made by milling, electroplating or micromachining

[3] – For milled moulds

2.3.3 Surface modification

Early work on silicon-glass and later polymer PCR microchips revealed the surface biocompatibility issue.[9, 103, 104, 105, 106, 107, 108, 109] For most microfluidic systems with biological applications, the surfaces are modified toward minimizing non-specific interactions, especially for proteins and cells. Proteins tend to adhere onto hydrophobic surfaces. Native silicon and most of the commodity polymers available are hydrophobic.[91] For a surface to be effective at protein rejection, the surface coating must be heavily hydrated, hydrophilic or neutral, densely packed, and conformational mobile. Neutral surfaces minimize electrostatic interactions, while highly hydrophilic surfaces minimize hydrophobic interactions. Hence, neutral and hydrophilic polymers have minimal or weak interactions with most globular proteins.

The surfaces of the microchips are important for microchip functionality and uniform surface treatment of complex shapes and geometries are therefore essential for microfluidic systems and for the biomedical applications therein. Surface modifications can be divided into two broad categories: 1) chemically or physically altering the atoms or molecules in the existing surface (*e.g.*, plasma activation and laser ablation), or 2) coating the existing surface with a material having a different composition.[110] A major challenge in surface modification is precise control over functional groups. Many surface modifications schemes produce a spectrum of functional groups such as hydroxyl, ether, carbonyl, carboxyl, and carbonate, in contrast to the intended functional group. However, charge density and charge location can be controlled in some degree by several parameters including (1) choice of polymer material, (2) fabrication protocol and (3) various surface treatments. Stability of surface chemistries and structures can change over time in response to the external environment. The driving force for these surface changes is the minimization of the interfacial energy.[110] As a result of unspecific adsorption, the surfaces capture compounds from solution passing through the channels, changing their concentration in solution. Any molecules deposited on the wall of the channel will also change the character of the surface. Analyte adsorption is a parameter that is highly dependent on several material characteristics including hydrophobicity and surface charge.[90] A specific concern for material used in optical devices is that the surface modification does not induce cloudiness or haze into the material.

Coatings used to modify the surfaces are mainly categorized as static or dynamic.[62, 111, 112] The static coatings can be covalently bonded to the surface, or physically

adsorbed relying on either hydrophobic interaction or hydrogen bonding or combinations thereof. Static coatings are applied in the fabrication of the chip before starting the microfluidic assay. For most silicon microchips a thin layer of silicon dioxide (SiO_2), which will function as a static coating, is deposited on the surfaces. Several publications have shown that SiO_2 layers are sufficient for enhancement of *e.g.* PCR.[9, 103, 104, 113] Silanization (*e.g.* with SigmaCote™) is another widely used process to prevent adsorption in silicon/glass microchips.[103, 113, 114] Of interest is that though silanization has been successfully applied to microfluidic devices, criticisms regarding the reproducibility of such coatings have been argued.[113]

Proteins such as bovine serum albumin (BSA) adsorb physically on to a large range of materials and can be used in microfluidic chips to make biocompatible surfaces.[115, 116] BSA is often used as a blocking agent to prevent non-specific binding within common biological assays such as for immunoassays (*e.g.* enzymed-linked immunosorbent assay, ELISA). Another approach of surface passivations is by using polymers such as polyethylene glycol (PEG),[113, 117] polyacrylamide, parlyene[69, 118] *etc.* PEG coated surfaces are one of the most successful ways to resist protein adhesion and biological attack. PEG is also known as polyethylene oxide (PEO), polyoxyethylene (POE) and polyoxirane.[119] PEO is the same polymer as PEG, but PEO typically signifies a larger molecular weight. PEG refers to molecular weights of less than 25 000 g/mol. PEG molecules can exhibit many forms. They can be linear or highly branched polymers, be covalently bound or physically adsorbed. PEG appears to be the most mobile, the most dynamic and the least interactive of all neutral and hydrophilic water-soluble polymers readily available.[120] The ability of PEG coated surfaces to prevent proteins and other biomolecules to adsorb at the surfaces are probably due to its unique solution properties and molecular conformation in aqueous solution.[121] It has an inert character which exposes uncharged hydrophilic groups and show very high surface mobility.[120] PEG will exhibit non-polar conformations near hydrophobic surfaces, leading to a more densely coated material, and will exhibit polar conformations far from the hydrophobic surface.[122] Thus, PEG surfaces usually consist of long PEG chains that protrude out from the insoluble surface into an aqueous solvent. Proteins and other biomolecules are prevented from approaching a PEG-coated surface because of an enhanced steric stabilization force. There are two main contributors to this repulsive force: an excluded volume component and a mixing

interaction component. The former is an elastic response from the loss of conformation entropy. When a protein gets close to a PEG-covered surface, the available volume for each polymer is reduced, and consequently, a repulsive force is developing owing to loss of conformational freedom of the PEG chains. The second is the osmotic interaction between the protein and the PEG-covered surface. In this case, the number of available conformations of PEG segments is reduced owing to either compression or interpenetration of the protein chains generating an osmotic repulsive force.[119, 121]

Dynamic coatings are introduced with the sample in the microsystem.[62, 64] Presumable, these substances will spontaneously migrate and adsorb to the inner surface of the microchip and prevent binding by components of the sample or reagent mixture. The most frequently used dynamic coating include proteins such as BSA[115, 123, 124, 125, 126] polymer solutions (PEG, polyvinylpyrrolidone (PVP))[107, 108, 109, 113, 126] and the non-ionic surfactant Tween 20. BSA is often included into reaction solutions to stabilize polymerase enzymes in addition to reduce undesired adsorption of the polymerase onto the inner surfaces of the reaction chamber. Excess enzyme often serves the same function as BSA, providing additional protein which stabilizes the enzyme and balances any negative effects arising as a result of enzyme interaction with solid surfaces and/or air-liquid interface. BSA may prove to be insufficient if the volume of the reaction chamber is in the low-microliter to nanoliter scale.[113] Dynamic passivation using polymers and proteins are attractive because they are inexpensive, and the coating procedure adds no additional steps into either microchip fabrication or the overall assay procedure.

It is important to note that these two passivation methods are not mutually exclusive. Hybrid coatings combining dynamic and static coatings are often used.[62, 64] For instance, combinations of silanization-BSA,[127] SiO_2-BSA,[105] SiO_2-PEG/PVP[109] as well as BSA-BSA[115] have been demonstrated. Covalent bonded coatings exhibit longer lifetimes than the physically absorbed dynamic coatings. However, dynamic coatings are usually easier to prepare.

In the present work, BSA has been used as dynamic coating in all chips. In addition, SigmaCote™, SiO_2 and PEG have been used for surface coatings.

2.4 Reagents on microchips

2.4.1 Inhibition and contamination

The main disadvantage of all amplification assays is the susceptibility to contamination of samples. The accuracy of nucleic acid based assays depends on the awareness of risk factors and the proper use of procedures for prevention of contamination. Reaction inhibition can be total or partial, and can become visible as complete reaction failure or as reduced sensitivity of detection. Inhibition of nucleic acid amplification reactions is typically caused by inhibitors interfering with the cell lysis necessary for extraction of nucleic acids, by nucleic acid degradation or capture and inhibition of enzyme activity. Inhibitory factors include organic and inorganic chemicals, detergents, antibiotics, buffers, enzymes, polysaccharides, fats and proteins.[128] However, their modes of action are not yet fully understood. Sources of contamination are diverse including water, reagents, disposables, equipment, sample carry over, amplicons, and environment.[128, 129]

False negative results may be obtained if clinical samples contain substances which inhibit the amplifications enzymes. It is, therefore, of extreme importance to try to reduce the amount of inhibition in these assays trough proper sample preparation. The quality of the assay depends on how the sample is taken, and how the sample material is handled and processed before analysis takes place. It is of importance that the targets of interest are not destroyed before performing the analyses. In most cases, the sample preparation step is the most difficult part of the procedure and the end result will strongly depend on the quality of this step. Therefore, it is crucial to inactivate enzymes like RNases and DNases in the cell sample during sample preparation as these enzymes will degrade the sample and subsequently inhibit the amplification.[130, 131] Equipment used in reagent preparation or production of microchips should be treated aseptically in order to minimize the chance for contamination and inhibition due to these enzymes or other compounds influencing the amplification. Cross-contamination caused by as few as only one contaminating amplicon or organism in the reaction mixture can produce false positive results. Suboptimal reaction conditions can influence the result obtained. By including both positive and negative controls, the integrity of the results of the assay will be maintained.

Miniaturization of the reaction volumes changes the environment in contact with the reagents, causing phenomena (*e.g.* adsorption) which are not a problem in the conventional microtitre plates. Accordingly, adsorption of crucial reagents from the reaction mixture must be prevented.

2.4.2 Adsorption of proteins

As mentioned earlier, protein adsorption is a major problem in microchips. When proteins in solution are in contact with another phase with which it is immiscible, protein molecules tend to accumulate at the interfaces between the two phases.[132] Protein adsorption is triggered by chemical and physical phenomena related to the surface materials and the surrounding medium in contact with them. The reduction in interfacial free energy is the main driving force for adsorption. Biocompatible materials reduce protein adsorption by minimizing the interfacial free energy between the surface and the solution.

Globular proteins are more or less spherical, with molecular dimensions in the range of a few to a few tenths of a nanometer. Proteins consist of amino acids which exhibit a wide variety of side chains which can be acidic, basic and have large variation in polarity. The protein itself has loops, tails, helices, and sheets that can make their way through coating layers and interact with the substrate below, Figure 7.[120]

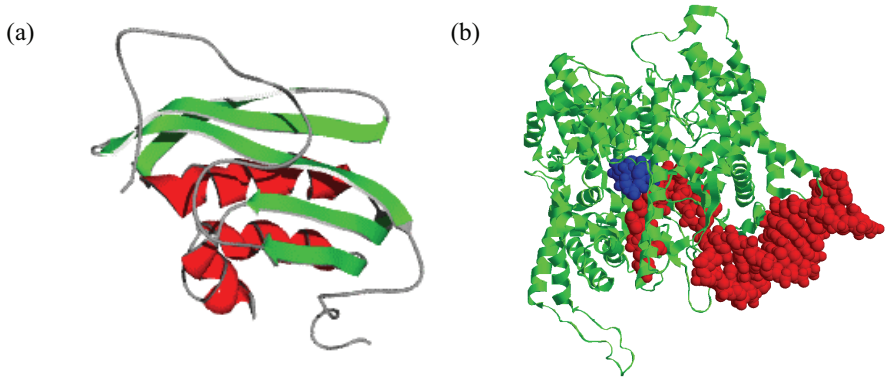

Figure 7 Proteins contains loops, tails, helices and sheets. (a) HIV RNase H. The green arrows represents sheets, red is helices and the grey is loops and tails.[133] (b) T7 RNA polymerase. The T7 RNA polymerase (green molecule is bound to a dsDNA (red) and producing mRNA (blue).[134]

The hydrophobic effect is considered to be the major driving force for the folding of globular proteins. It results in the burial of the hydrophobic residues in the core of the protein, shielding these groups from contact with water. Charged groups are predominantly found on the surface of the protein in contact with water. However, the smaller the protein molecules are, the larger the deviation from sphericity it will be. Small protein molecules are more often asymmetrical and tend to have a relatively more hydrophobic surface than larger and more spherical molecules.[132] In general, hydrophobic areas on globular proteins are small and limited in area.[121] The groups on the surface of a protein are the ones most likely to interact with a solid surface, although interior groups might be exposed through conformational changes. Proteins adsorb to most interfaces due to a large repertoire of intermolecular interaction between proteins and surfaces.[120] The major interactions that drive the interfacial activity and adsorption of proteins are the water structure-driven hydrophobic effect, electrostatic interactions, and strong hydrogen-bonding interaction characterized by cooperative, multiple hydrogen bonds.[120] It has been reported that hydrophobic surfaces adsorb more protein than hydrophilic ones, and that dehydration of hydrophobic surfaces promotes protein adsorption from aqueous solution.[135] It is assumed that protein adsorption is related to the number and size of the hydrophobic patches on the protein's exterior and that the surface adsorption of proteins increases with hydrophobicity and size. Transferring a protein molecule from an aqueous solution to an interface involves a change in the environment, and this process may induce structural rearrangements.

Electrostatic repulsion between surface and protein does not always prevent adsorption. Charge antagonism can effectively be annihilated by co-adsorption of low-molecular-weight ions from solution. The complex surfaces of proteins in combination with the fact that many real surfaces are heterogeneous, complicates the prediction of how a protein will interact with a surface.

Protein adsorption takes place at a timescale of seconds to a few minutes.[120, 136] Changes in conformation can occur immediately upon adsorption, but time-dependent conformational changes also occur. Orientation of proteins on the surface can vary. Protein adsorption is often apparently irreversible or only partially reversible by dilution, although there are examples of reversible adsorption. The desorption process depends upon the incubation time of the protein with the substrate: the longer the incubation time, the slower the

desorption.[137] The desorption can be affected by changing the pH, increasing the ionic strength or by introduction of a complex agent.[138] Although the protein may be irreversibly adsorbed with respect to dilution, it still can be exchanged by protein molecules in solution, or by low molecular-weight compounds. The amount of a protein that a given surface will adsorb depends on the solution it contacts, especially the protein content of that solution, the amount of other proteins present, the history of the surface with respect to protein contact, as well as conditions such as flow.[139] The protein-surface interaction appears to contain a large number of time-dependant or dynamic phenomena in addition to normal kinetic constrains caused by the diffusion of protein molecules to the solid surface.[140]

2.4.3 Protectants and reagent stability

In this work, NASBA reagents from the PreTect™ HPV-proofer kit (NorChip AS) were used. The kit contains all reagents needed to perform an amplification reaction. It consists of lyophilized reagent spheres, lyophilized enzyme spheres with their respective diluents in addition to a stock solution of KCl. Lyophilization is considered one of the best methods for stabilization of certain reagents for long-term storage. The lyophilized spheres need to be dissolved before reaction. In order to make a self-contained microchip, which ensure long-term stability of the NASBA reagents, the dissolved reagents needed to be spotted and dried in the reaction chambers on the microchip. However, it is not possible to dry all the reagents in the NASBA mixture. Thus, these reagents have to be introduced into the reaction mixture through the sample. To stabilize the enzymes during the drying step on the microchip is considered the most critical step in the process.

The NASBA amplification technology depends on the simultaneous activity of three different enzymes: AMV-RT, RNase H and T7 RNA polymerase.[29] Shortly, AMV-RT is a RNA-dependent DNA polymerase that catalyzes the polymerization of DNA using template DNA, RNA or RNA:DNA hybrids. The molecular weight of the avian enzyme is 160 kDa and the enzyme consists of two polypeptide chains.[141] The enzyme requires a primer (DNA primers are more efficient than RNA primers) as well as Mg^{2+} or Mn^{2+} for polymerization of DNA, and it possesses an intrinsic RNase H activity. AMV-RT is optimal at 42°C, however, it is stable at higher temperatures (37 – 58°C) as well. The optimum pH for the avian enzyme is

8.3. The length of the cDNA synthesized by AMV-RT is greatly reduced when reactions are carried out at a pH that differs from the optimum by as little as 0.2 units.

RNase H is a non-specific endoribonuclease which hydrolyses the phosphodiester bonds of the RNA moiety in DNA:RNA hybrids. A minimum of 4 base pairs (bp) in a RNA:DNA hybrid is required for activity. The enzyme does not hydrolyze single- or double-stranded DNA. RNase H is a monomer of 17.6 kDa[141] which contains two domains, one of which has a Mg^{2+}-binding site enmeshed in β-strands. The enzyme is inactivated after 20 minutes at 65°C.

T7 RNA polymerase is a DNA-dependent RNA polymerase that recognizes and initiates synthesis of RNA on double-stranded DNA templates that carry the appropriate T7 specific promoter. The RNA polymerase has extremely high specificity for its 25 bp cognate promoter sequence. The T7 RNA polymerase has a molecular weight of 107 kDa[141] and the optimal activity is in the pH range of 7.7 – 8.3. Full activity requires Mg^{2+}, a DNA template, T7 promoter and all rNTP. The T7 RNA polymerase gives rise to 100 – 1000 specific RNA molecules.[39, 40] The RNA produced when using the T7 RNA polymerase is biologically active as mRNA.

Macromolecules, especially proteins and polypeptide-containing compounds commonly exist in their native state as a complex, three-dimensional folded structure, the tertiary structure. Often, the activity of the enzymes is critically dependent on the tertiary structure and is severely reduced or even eliminated if the structure is denatured. Enzymes are usually unstable in aqueous systems at room temperature and needs to be stored frozen. As mentioned previously, lyophilization is also considered to be one of the best methods for stabilization of fragile enzymes for long-term storage. During lyophilization water is removed from a frozen sample by sublimation and desorption. However, the lyophilizing process of *e.g.* enzymes is not trivial. The enzyme structures are easily distorted during the freezing and drying processes. Cryoprotectants can protect the enzyme from denaturation during the freezing process, while lyoprotectants can prevent protein inactivation during drying. By mixing stabilizing protectants with the enzymes of NASBA before spotting and drying, improved long-term storage stability is possible.[142]

Cryoprotectants protect molecules against stress such as shearing, caused by the formation of crystals during the freezing process. Cryoprotectants protect largely by

preventing large ice-crystals from forming. Commonly used cryoprotectants are DMSO, sorbitol and glycerol. However, it has not been possible to employ these compounds in this work, since they tend to form a hard crust instead of a powder during drying. The NASBA reagents include both DMSO and sorbitol; however, these compounds have to be added in the reaction mixture together with the sample.

PEG is another cryoprotectant widely used. It stabilizes enzymes during freezing, due to preferential exclusion of PEG from the enzyme's surface because of steric hindrance.[143] Increasing the concentration of the protectant will increase the stability of the enzyme during freezing. However, PEG is an extremely strong protein precipitant and should not be added in too high concentrations.

Lyoprotectants stabilize and support enzymes during the drying process. In general, drying results in a decrease of both α-helix and random structures and an increase in β-sheet structures.[142] Lyophilization in the absence of stabilizers has been observed to induce significant conformational changes on enzymes.[144] The most common lyoprotectants are sugars (*e.g.* trehalose, sucrose, lactose) and polyols (*e.g.* mannitol). While trehalose seems to be the most commonly used lyoprotectant, other compounds like sucrose, mannitol, and lactose are also effective. The amount of trehalose necessary to preserve activity is proportional to the concentration of enzyme.[145] It is possible to preserve sensitive macromolecules by drying at ambient temperature and at atmospheric pressure in the presence of trehalose. The unique properties of trehalose in preserving the structure and function of proteins such as enzymes and antibodies, and other macromolecules in the dry state, is due to hydrogen bonding of trehalose molecules via their hydroxyl groups, to appropriate groups on the macromolecule. Trehalose substitutes the structural (bound) water molecules so that there is no collapse of macromolecular structure upon drying.[146]

Polymers such as PVP and BSA have been reported to protect multimeric enzymes against inactivation by inhibiting dissociation during freezing and drying.[147] The polymers can stabilize the quaternary structure by inhibiting dissociation in the frozen solution, during the initial phase of the sublimation step of lyophilization.

In the present work, PEG, trehalose, PVP as well as BSA have been tested with regard to inhibition of the NASBA reaction and preservation of enzymes during the drying process.

2.4.4 Storage of reagents on microchips

For fully automatic μTAS devices without protocols, it is required that all reagents necessary for complete analysis can be stored on the microchips for a prolonged period of time. At present, the reagents are typically introduced in the reaction chamber via interconnections from *e.g.* large syringes, pumps or by larger local reservoirs on chip.[115, 148] However, a couple of new storage microfluidic cartridges preloaded with wet reagents have been reported.[55, 149, 150, 151] By drying and storing the reagents directly in the reaction chambers of the microchips, one can reduce handling time and contamination risk. So far, only a few examples of microfluidic systems have been described with dried proteins incorporated in a microchip.[152, 153] Storage of reagents on microchips require several process steps; dissolution of lyophilized reagents, spotting, drying, sealing and storage.

The easiest way to introduce reagents on chip is by applying the reagents as liquids. As precise dispensing of liquids in the range below one microliter has become increasingly important in the chemical, biological and pharmacological industries, several devices are available. The driving force for the development of technology for spotting of small volumes has come through the expansion of the microarray field.[154, 155, 156] More recently, new methods and devices for reagent dispensing have been developed to meet the increasing interest in miniaturization of biological and chemical assays. These testing platforms demand precise metering of the smallest amounts of reagents on planar substrates or in cavities. Today very sophisticated spotting systems can create regular arrays or arbitrary spotting patterns of many thousands of different substances on an area of a few square inches, depending on the instrument used. Commercially available dispensing robots typically can dispense volumes of 50 nl or more per droplet or dispense cycle. Microfluidic devices, on the other hand, may be able to support fluid volumes even within the smaller picoliter range.[157, 158, 159, 160, 161, 162]

Two main spotting techniques are presently available; contact spotting and non-contact spotting. The contact spotters are based on pins, rings or tips. The printing head characteristics determine the probe spot quality and reproducibility.[163] In addition to the properties of the spotted liquid, the hydrophilic or hydrophobic properties of the substrate determine the size and the shape of the spot. The drawbacks of pen-like devices for patterning surfaces are the lack of control after deposition of the material, problematic drying and mechanical wear.[44] Contact spotters are useful in dispensing volumes from typically slightly under a nanoliter to

several nanoliters.[164] Additionally, the physical contact between the metallic (or composite material) spotter tips and the surface can denature delicate proteins and are therefore in many cases not recommended for the spotting of proteins.[165] Such spotters can also damage the surface via physical scratching. Nanoliter spots have a tendency to evaporate quickly. To minimize such effects, glycerol might be added to protein solutions during the spotting step. However, while this solution minimizes the evaporation effects, it dramatically increases the viscosity of the sample solution leading to surface tension effects that might make delivery difficult.[165] Capillary and adhesive forces at the tip can result in large errors when moving into the nanoliter range and provide a risk of cross-contamination.

The non-contact spotters are based on solenoid, piezo, ink-jet, microfluidic devices and laser principles. Non-contact spotters give a high level of reproducibility since the droplets spotted always have the same size, and it is therefore easier to dispense reagent solutions of different composition.[164] Some of these spotters are also capable of making very small droplet sizes, even in the picoliter range.[157, 164] Aqueous reagent solutions can easily be positioned at substrates with hydrophobic areas due to the surface tension obtained on the hydrophobic surface.[163] The spotted aqueous droplets will only marginally spread out. Non-polar solutions, on the other hand, will wet hydrophobic surfaces quite well. However, such surfaces are not preferred in all cases as proteins tend to adsorb to hydrophobic surfaces and become inactive. The spreading on hydrophilic surfaces will also depend on the wetting properties of the spotted reagents. Non-contact spotters are generally recommended for the spotting of proteins on surfaces.[161] Such spotters have low mechanical load on the liquid to be spotted and as a result, the kinetic energy of the delivered liquid is low.

Several methods exist for drying biological material. Air drying and freeze-drying are two commonly used methods. Air drying is the simplest form of drying at ambient pressure. However, it usually requires elevated temperature or long periods of time, and the effects of surface tensions and the long timescale over which drying occurs, tends to result in severe irreversible denaturation of sensitive biological reagents. Freeze-drying is the method of choice for preserving biological and pharmaceutical products.[142, 154] The water content is reduced to values that will no longer support biological activity or chemical reactions. Furthermore, the porous structure of the ‘cake’ achieved by freeze-drying allows for extremely rapid reconstitution of the sample. Other drying methods may produce an impermeable ‘skin’

in the top layers of the dried reagents. This film may trap excessive residual moisture, which can lead to instability and decay. Although, freeze-drying is considered the gentlest drying method for preservation of biological material, it was not suitable for the present work due to the spotting procedure. Instead air drying was employed.

Water evaporates rather quickly, even at standard room temperature and humidity. The evaporation is critical when the spotted droplets of reagents are in the nanoliter range. Evaporation itself is a complex process. The dynamics of the drying processes for single-component systems are mostly determined by the internal cohesive energy of the droplets, irrespective of the substrate surface and droplet size. In most applications, the droplets also have different kinds of dissolved molecules, which may contribute to the drying dynamics.[166, 167] Two processes are included in drop evaporation: diffusion of liquid molecules into the air (diffusion part) and flow of the liquid molecules from inside the drop to the free outer shell liquid layer within the liquid-vapour interface (evaporation part). The diffusion part remains steady during drying and is not sensitive to the variation of temperature. The evaporation part, however, is an active factor and determines the differences in drop evaporation behaviours.[167]

In the absence of water molecules, the side chains of the amino acids interact with each other and 'lock up' the conformation. This deprives the enzyme molecule of the flexibility that is necessary for its catalytic activity. The enzyme activity depends on the ionization state of the active-site residues. Upon rehydration, an enzyme which is in a native conformation in the dried state exchanges the water substitute (protectants) for water and remains in the native state. Enzymes that are unfolded in the dehydrated state and do not refold properly upon rehydration, looses activity. Unfolded enzymes often have a tendency to aggregate.[168] Water 'deposition' follows a definite sequence of events. First it gets deposited on charged and polar amino acids, and then around the hydrophobic clusters.

2.5 Microfluidics and actuation

Microfluidics is the study of transport processes of fluids in microchannels. Typical channel diameters are of around ten to several hundred micrometers, which facilitate handling and analysis of volumes significantly smaller than a nanoliter. Microfluidic chips are the primary

element of most LOC devices and μTAS. The chips may consist of valves, mixers, pumps, filters and heat exchangers *etc.* The components allow metering, dilution, flow switching, particle separation, incubation of reagents, and sample dispensing or injection. Due to these components functionalities, both continuous-flow and droplet-based (plug-based) microfluidics are possible.[169] There is a wide range of methods to generate fluid flow in microfluidic devices, including the use of electricity (electroosmosis, electrophoresis, dielectrophoresis, electrowetting), mechanics (syringe or vacuum pumps, thermopneumatic pumps, membrane actuated pumps, centrifugal force, ferrofluidic plugs), magnetism (magnetohydrodynamic pumps), capillary effects or fluid motion due to evaporation and osmosis *etc.*[85,89, 170]

The fundamental physics of fluidic systems change dramatically as the volume decreases. On the microscale level, other forces than those experienced in every day life become dominant. On small scales, inertia is insignificant, while viscosity becomes very important. Thus, microfluidic devices exhibit almost exclusively laminar flow, and depend solely on diffusion as transport mechanism of molecules. Another characteristic feature of microfluidics is the dominance of surface effects due to the large surface area-to-volume ratio on the micrometer scale. Gravity can almost always be neglected on microscale. For such reasons, downscaling existing large devices and expecting them to function well at the microscale is often counterproductive.

2.5.1 Diffusion

In a liquid or gas, all molecules move by Brownian motion in all directions, as long as no external forces are applied. Each molecule moves in certain directions for a certain time, until it hits another molecule, after which it changes direction. Because the molecules are indistinguishable from one another, no net flow is observable. Diffusion is a transport phenomenon being the spontaneous spreading of matter (molecules), heat or momentum. A solution in equilibrium will seek uniform distribution of its molecules; generating concentration gradients through the solvent. The net movement of molecules will be from the area of high concentration to the area of low concentration. The statistical movement of a single molecule in a fluid in two dimensions can be described as in, Equation 1.[88]

$$x = \sqrt{2Dt} \qquad 1$$

where x is the average distance moved after an elapsed time t between molecule collisions, and D is the diffusion constant which depends on the size and shape for a given molecule. The diffusion coefficient for a solid spherical particle is given by Equation 2:[171]

$$D = \frac{kT}{3\mu\pi d} \qquad 2$$

where k is the Boltzmann constant, T is the absolute temperature, μ is the dynamic viscosity and d is particle diameter. A large diffusion constant means fast movement. In general, the larger a molecule is, the smaller is its diffusion constant. The dimension of a microchannel has a large influence on the diffusion and mixing of liquids in a channel. Table 3 shows characteristic diffusivities of typical species used in microsystems.

Table 3 Typical diffusivities for various species in water at room temperature.[172]

		Characteristic diffusivities			
Species	**Typical size**	**Diffusion constant, D [μm²/s]**	**Time of diffusion 10 μm [s]**	**Time of diffusion 100 μm [s]**	**Time of diffusion 1 mm [s]**
Solute ion	0.1 nm	2×10^3	~0.025 sec	~2.5 sec	~4 min
Small protein	5 nm	40	~1.25 sec	~2 min	~4 hours
Virus	100 nm	2	~25 sec	~42 min	~3 days
Bacterium	1 μm	0.2	~4 min	~7 hours	~4 weeks
Mammalian/human cell	10 μm	0.02	~42 min	~3 days	~41 weeks

Mixing is one of the challenges in microfluidic devices due to the absence of inertial effects (turbulence) on microscale flows. All strategies to improve mixing have examined the possibilities to either reduce the diffusion distances or to agitate the flow. Strategies to improve mixing include splitting streams into smaller streams and folding and relaminating the streams again and again, thereby again minimizing the diffusion distances by increased interfacial contact. Another strategy for mixing is through chaotic advection. Here unique channel designs, including bas-relief structures on channel floors and 3-dimentional serpentine microchannels may be used to induce chaotic advection.[173] Thus, chaotic flows may occur

under certain rare conditions, when geometries change rapidly and do not allow the flow to reach a steady state before the next change in geometry. A third method is the employment of active mixers, which use external energy to induce temporary fluctuations. In contrast to the turbulent flows in macroscale, chaotic flows on microscale are quickly damped.

2.5.2 Surface tension and contact angle

Surface tension (γ) or interfacial energy is energy per area of an interface between two phases, whether they are solids, fluids or gases. Surface tension is caused by the attraction between the molecules of the fluid interface due to various intermolecular forces. The molecules prefer to be in the interior where the highest number of bonds is possible. In the bulk of the liquid, each molecule is pulled equally in all directions by neighbouring liquid molecules, resulting in a net force of zero (Figure 8a). At the surface of the liquid however, the molecules are surrounded by fewer neighbours, and the liquid tends to minimize the number of "broken" bonds by minimizing the surface area. All molecules at the surface are therefore subject to an inward force of molecular attraction which can be balanced only by the resistance of the liquid to compression. The lack of chemical bonds results in a higher energy for the surface molecules. Therefore, the liquid surface will tend to minimize its surface area, often resulting in curved surfaces.

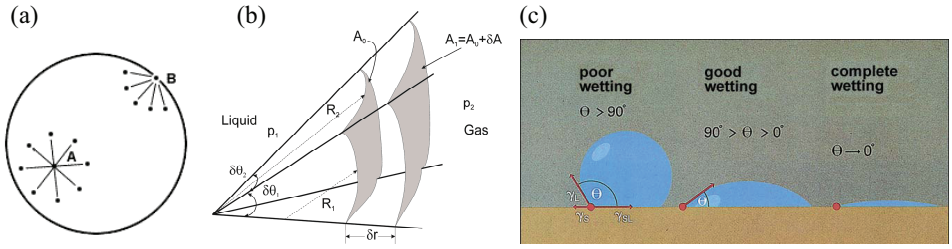

Figure 8 (a) Schematic drawing to illustrate the surface tension caused by intermolecular forces acting between molecules at the liquid/gas interface and in the liquid bulk. A – The bulk molecules are pulled equally in all directions by the neighboring liquid molecules. B – The surface molecules are subjected to an inward force of molecular attraction to compensate for the lack of chemical bonds in the direction of the gas phase.[174] (b) Differential expansion of a small section of a liquid/gas interface with locally constant curvatures. (c) The contact angle (θ) is defined as the angle between the solid-liquid and the liquid-gas interface at the contact line. The wettability of a liquid on a surface can be described by the contact angle.[174]

The pressure difference built up across the interface is balanced by the intermolecular forces. An expression for the pressure difference can be derived[138] by considering the energy required to expand a curved surface, A, (Figure 8b). This pressure difference, Δp, is known as the Young-Laplace equation (Equation 3):

$$\Delta p = \gamma\left(\frac{1}{R_1}+\frac{1}{R_2}\right) \qquad 3$$

where R_1 and R_2 are the radius of the curvature. Δp is defined positive and is thus the pressure of the concave side minus the pressure of the convex side.[138]

Wetting is the contact between a liquid and a surface, when the two are brought into contact. Chemical affinities between a surface and a liquid at the molecular level determine the wettability of a surface and the resulting shapes of liquid drops. When a liquid has a high surface tension (strong internal bonds), it will form a droplet, whereas a liquid with low surface tension will spread out over a larger area (bonding to the surface). On the other hand, if a surface has a high surface energy, a drop will spread out, or wet, the surface. If the surface has low surface energy, a droplet will form. This phenomenon is a result of the minimization of interfacial energy. If the surface is high in energy, it will tend to be covered with a liquid because the interface then formed will lower its energy.

The contact angle (θ) is defined as the angle (measured inside the liquid) that is formed on the junction of the three phases, at the solid-liquid-gas junction, as depicted in Figure 8c. It can be expressed as in Equation 4 and is known as Young's equation.[138]

$$\gamma_L \cos\theta = \gamma_{SG} - \gamma_{SL} \qquad 4$$

where γ_L is the liquid-gas interfacial energy, γ_{SG} is the solid-gas interfacial energy and γ_{SL} is the solid-liquid interfacial energy. The contact angle is a result of the static equilibrium of the minimum interfacial energies of all phases. A contact angle of 90˚ or larger generally characterizes a surface as not-wettable, and one less than 90˚ means that the surface is wettable. In the context of water, a wettable surface may also be termed hydrophilic and a non-wettable surface hydrophobic. For a hydrophobic surface, the hydrophobic effect is

dominant. The hydrophobic effect is the property that causes electrically neutral and non-polar molecules to self-associate in the presence of aqueous solution. Matter seeks to be in a low energy state, and bonding reduces chemical energy. Water is electrically polarized, and is able to form hydrogen bonds internally. However, water molecules are incapable of forming hydrogen bonds to non-polar molecules (*e.g.* alkanes, hydrocarbons, fluorocarbons and inert atoms), therefore water repels the hydrophobic molecules in favour of bonding with itself. Contact angles can often be changed by chemically modifying surfaces or by addition of certain solute molecules into the medium that adsorb on the surface. The contact angle is independent of the surface geometry. However, the contact angle is not always stable and static. Contact angle hysteresis is a common phenomenon and arises when a three-phase boundary becomes trapped in transit, lacking sufficient energy to surmount the energy barrier to a lower energy state. In this case, it is generally observed that the contact angle on a liquid advancing is different from the receding on a surface. Contact hysteresis is not fully understood, but is generally attributed to surface roughness, surface heterogeneity, liquid-surface interactions or due to a dynamic contact angle.[138]

2.5.3 Capillary forces

The two concepts, contact angle and surface tension, is the basis for understanding the capillary forces that act on liquids inside microchannels. The dynamics of surface tension-driven fluid follow from a balance of capillary and viscous forces.[172] The surface tension along with the dimensions and the geometrical angles of the microchannels determine how strong the capillary forces are, and can lead to pressure gradients in the liquid. The pressure difference of the meniscus causes flow transport, so it is similar in many ways to pressure-driven flows.[8] Variations of the Young-Laplace equation (Equation 3) makes it possible to calculate the pressure differences across the menisci of liquid plugs in microchannels. The equations depend on the cross sectional shapes (*e.g.* circular or rectangular) of the microchannels. In Figure 9a, the liquid-gas interface in a circular microchannel is illustrated. The curvature of the interface, R, can in this case be expressed as $R= r/\cos\theta$, where r is the radius of the microchannel. In a similar manner can the curvature for a rectangular shaped cross section be expressed as $R=(1/h+1/w)/\cos\theta$, where h and w is the height and width of the channel, respectively. Figure 9b presents a liquid plug in a microchannel, where the two

menisci of the plug are equal in size and the plug rests in equilibrium. The pressure difference across the two menisci in Figure 9b is zero. A liquid plug that is not in equilibrium is shown in Figure 9c. The pressure difference across the two menisci in this case is non-zero. The pressure is higher on the right side meniscus than the left, resulting in a force that will pull the plug towards the right. In Table 4, some variations of the Young-Laplace equation (Equation 3) for channels with rectangular and circular cross sections are given. The cross section of the microchannels used in the present work was rectangular.

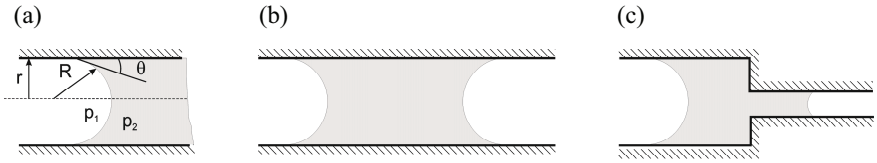

Figure 9 Liquid-gas interfaces in microchannels. The liquid is coloured grey. The cross section of the microchannels might be either circular or rectangular. The radius, r, of a circular cross section is shown in (a). The height, h, and width, w, of a microchannel with a rectangular cross section are not shown. The width is perpendicular to the text plane. (a) The driving force a liquid plug. (b) Liquid plug in equilibrium. (c) The liquid plug is not in equilibrium.

Table 4 A variation of the Young-Laplace equation (Equation 3) for channels with different cross sectional shapes.

Case	Circular	Rectangular
One meniscus	$\Delta p = \frac{2\gamma\cos\theta}{r}$	$\Delta p = 2\gamma\cos\theta\left(\frac{1}{w}+\frac{1}{h}\right)$
Two meniscus	$\Delta p = 2\gamma\cos\theta\left(\frac{1}{r_1}-\frac{1}{r_2}\right)$	$\Delta p = 2\gamma\cos\theta\left[\left(\frac{1}{w_1}+\frac{1}{h_1}\right)-\left(\frac{1}{w_2}+\frac{1}{h_2}\right)\right]$

The capillary pressure along a flow path can be altered by changing either the channel geometry or surface properties. Aqueous liquid held between hydrophobic walls forms convex surfaces which result in elevated pressure inside the liquid plug.[170] Non-wetting areas arrest capillary intrusion. In aqueous liquid plugs surrounded by hydrophilic walls, a concave surface is created and the pressure inside the plug is lowered. Each wettable ($\theta < 90^{\circ}$) wall of the microchannel contributes to generate a negative pressure front of the liquid and to draw aqueous liquid into the channel.[44]

Capillary flow can be disturbed by pinning of the liquid meniscus which often is caused by contact angle hysteresis. This uncontrolled effect prevents the exact prediction of the movement of the liquid front. In the case of pinning, the pressure drop in the meniscus is significantly affected.

In microsystems involving liquid plugs enclosed by gas, the effects of pressure changes due to heating of the system and evaporation of the liquid must be taken into account. The plugs can be displaced due to increased vapour pressure if parts of the channel system are closed. If the gas phase is not in equilibrium with the liquid phase, the liquid will evaporate until the gas phase is saturated with molecules from the liquid, which can result in shrinkage of the plug volume. These effects depend largely on the size of the dead volume.

2.6 Detection technology

The amplified single-stranded RNA transcripts of the NASBA reaction are ideal for use in a hybridization-based detection system with sequence-specific probes. The first two detection methods described in relation to NASBA were electrochemiluminiscense (ECL) and enzyme linked gel assay (ELGA), which both are endpoint analyses.[27, 34, 175] Today, the most widely used probes are fluorescent molecular beacons which hybridize to the amplicons during amplification, enabling real-time detection.[30, 31, 34, 176, 177, 178, 179] This cuts down the total analysis time, in addition to providing information about the kinetics of the reaction. In contrast to ECL and ELGA, no extra detection step is required when employing molecular beacons and the tubes can remain closed and carry-over contamination is therefore prevented.

Molecular beacons are short ssDNA molecules composed of a hairpin-shaped oligonucleotide that contains both a fluorophore and a quencher group, as depicted in Figure 10.[31, 180] The loop part of the molecule contains the sequence complementary to the sequence of the target nucleic acid, whereas the stem is unrelated to the target and has a double-stranded structure. One arm of the stem (5' end terminal) is labelled with a fluorescent dye, *e.g.* 6-carboxy-fluorescein (FAM), and the other arm (3' end terminal) with a non-fluorescent quencher *e.g.* 4-(4'-dimethylaminophenylazo) benzoic acid (dabcyl). The fluorescent dye serves as an energy donor and the non-fluorescent quencher plays the role of an acceptor. The stem holds these two moieties in close proximity of each other, causing the

fluorescence of the fluorophore to be quenched by energy transfer. When the hairpin structure is “closed”, the probe is unable to fluorescence. When the probe encounters a target molecule, the molecular beacon undergoes a conformational reorganization that forces the stem to open, because the loop hybridizes to the target molecule. This hybrid is longer than the stem, and therefore more energetically favourable. Furthermore, the fluorophore and the quencher are separated from each other, leading to the restoration of fluorescence.

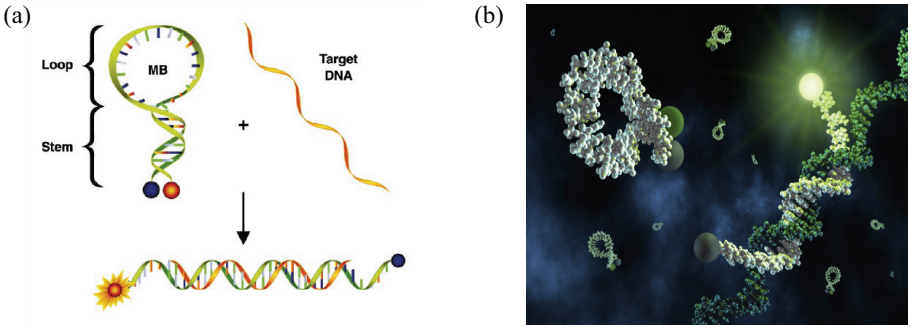

Figure 10 Conformational structure of molecular beacon probes. (a) Prior to hybridization the fluorescence is minimal due to the stem-loop structure of the molecular beacon), maintaining the fluorophore (red/yellow) and the quencher (blue) in close proximity leading to quenching.[178] When introducing the target to the molecular beacon, it undergoes a spontaneous conformational change forcing the stem apart. Consequently, the fluorophore and quencher separates and result in the restoration of the fluorescence. (b) Computer art of unhybridized stem-loop molecular beacons and a fluorescent hybrid. (bioMérieux, Marcy l’Etoile, France)

Two forms of energy transfer may take place in molecular beacons: direct energy transfer and fluorescence resonance energy transfer (FRET).[181] Direct energy transfer depends on *contact* between the fluorophore (donor) and quencher (acceptor). The collision between the fluorophore and the quencher changes the energy level of the excited fluorophore, resulting in quenching. The quenching moiety dissipates the received energy as heat.

The mechanism of FRET involves a donor fluorophore in an excited electronic state, which may transfer its excitation energy to a nearby acceptor chromophore (quencher) in a non-radiative fashion through long-range dipole-dipole interactions.[163] The theory supporting energy transfer is based on the concept of treating an excited fluorophore as an oscillating dipole that can undergo an energy exchange with a second dipole having a similar resonance frequency. The principle of FRET is described in Figure 11.

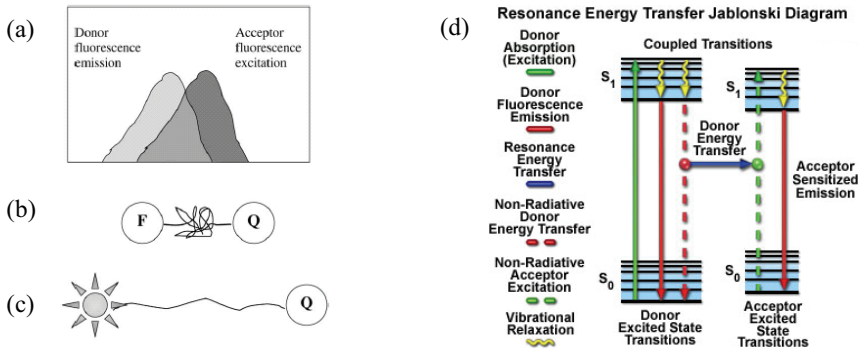

Figure 11 The FRET concept. (a) The overlapping spectra of the donor emission and the acceptor excitation, (b) with donor (F) and quencher (Q) in proximity, no fluorescent signal is generated, but (c) when separated, the donor is able to emit fluorescence uninhibited.[163] (d) The coupled transitions involved between the donor emission and acceptor absorbance in FRET. Absorption and emission transitions are represented by straight vertical arrows (green and red, respectively), while vibrational relaxation is indicated by wavy yellow arrows. The coupled transitions are drawn with dashed lines that suggest their correct placement in the Jablonski diagram should they have arisen from photon-mediated electronic transitions. In the presence of a suitable acceptor, the donor fluorophore can transfer excited state energy directly to the acceptor without emitting a photon (illustrated by a blue arrow). The resulting sensitized fluorescence emission has characteristics similar to the emission spectrum of the acceptor.

Molecular beacons in solution with their targets can exist in three states (Figure 12a): bound to target (phase 1), free of target in the form of a hairpin (phase 2), and free of target in the form of a random coil (phase 3).[182] Poor signal-to-background ratios may be caused by the presence of uncoupled fluorophores in the preparation, or by the presence of oligonucleotides that have a fluorophore, but not a quencher. It is therefore of importance to use pure molecular beacons. The fluorescence of a solution at a given temperature is the sum of the fluorescence of the molecular beacons in each of the three states. Figure 12b shows the thermal denaturation of a typical molecular beacon. At lower temperatures, the molecular beacons are in a closed hairpin state and do not fluoresce. However, when the temperature increases, the helical order of the stem gives way to a random-coil configuration, separating the fluorophore and the quencher, restoring fluorescence. When the molecular beacon is bound to the target, the molecular beacons fluoresce brightly at low temperatures, but as the temperature is slowly raised fluorescence decreases significantly, before it increases as the temperature increases.

The fluorescence curve obtained when using molecular beacon probes is dependent on two factors: the NASBA-driven time-dependent increase in RNA levels and the binding of the molecular beacon to this RNA. Time-to-positivity (TTP) is defined as the time in minutes when the fluorescence signal is above the background or no template signal.[179, 183]

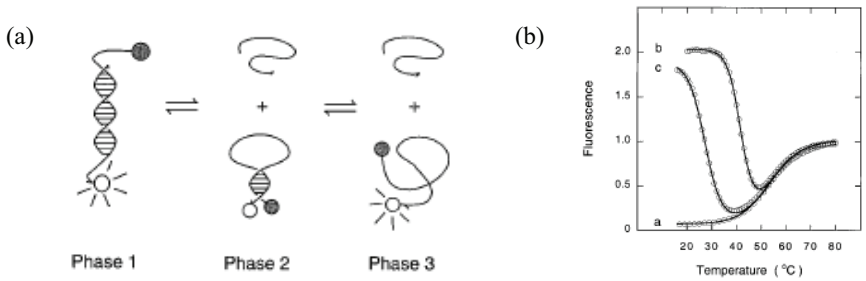

Figure 12 (a) Schematic representation of the three molecular beacon phases: bound to target (phase 1), free of target in the form of a hairpin (phase 2), and free of target in the form of a random coil (phase 3). (b) Thermal denaturation profile of solutions containing molecular beacons: trace a, in the absence of targets; trace b, in the presence of excess of perfectly complementary targets; and trace c, in the presence of an excess of mismatched targets.[182]

2.6.1 Fluorescence detection in microchips

The most commonly used detection method adapted to the microsystem technology is fluorescence detection. The advantages of fluorescence detection is superior sensitivity, high specificity enabling real-time measurements, and multiplexing capabilities.[163] Microsystem technology enables structures on the micrometer and nanometer scales, using these features it has been possible to achieve the requirements for the analysis and manipulation of samples on a single molecule scale.[46, 47, 184, 185] Despite the excellent sensitivity of fluorescent detection, it has some disadvantages. As most samples are not fluorescent, it is often necessary to fluorescently label the samples. The guided excitation light can also generate heat, which will increase the temperature of the region of measurement and could effect the analytical assay.[101] The ultimate sensitivity of optical detection is to a large degree dependent on the noise and drift over time caused by thermal expansion and vibration within the system.[186] Unless the microsystem contains an internal self-calibration of the fluorescent signal feature, it will be

difficult to obtain quantitative data.[187] Dye molecules tend to leach and photobleach as a function of time, and therefore it is hard to control their concentration. High intensity illumination accelerates photobleaching. By adding antifade reagents, photobleaching may be reduced.[188] Additionally, quenching of the fluorophores that result in non-emissive transition to the ground state is also possible. Interaction between the fluorophore and the many substances in the measured solution may also interfere with the measurment.[187]

Background noise is often the important limitation to ultrasensitivity. The background signals may originate from the sample constituents, or from unbound or non-specifically bound probe (reagent background), and scattering due to; reflection, refraction, luminescence from the optics, solutions or chip substrates. Optical noise is generated every time light is reflected or refracted from an optical interface. The surface roughness of the microfluidic chips is a critical parameter for optimal quality of the measurement. When the surface roughness is large, the excitation and emission lights in the measurements are scattered and the optical pathway is disturbed, and this may reduce the sensitivity of the system and generate crosstalk in a multichannel system. Light is also lost through reflection caused by changes in the refractive index at the surface between two materials. Considerations of assembling devices with direct contacting of refractive-index matching materials and components will minimize losses and generation of stray light at the various interfaces. Therefore, selecting the appropriate chip material is of importance. It must be transparent to light, specifically in the excitation and emission wavelengths. The material should exhibit no or minimal autofluorescence, which otherwise leads to a high background signal. Autofluorescence is generally higher for polymer materials compared to glass.[90] The autofluorescence of polymers can often be related to the intrinsic bulk polymer, additives, impurities or degradation products. The differences in magnitude of the initial fluorescence for similar polymers can also be explained by differences in the polymer production processes between vendors, and the post-production age and handling of material.[96] Stored polymers that have been exposed to light over a period of time can loose a significant fraction of its fluorescence compared to newly fabricated polymers. Thus, there is a huge potential for device-to-device variation among polymer chips.[96]

Another problem regarding the materials used in microfluidic chips is that they are transparent in the visible region and have higher refractive indices than that of air or the

surrounding environment. Therefore, the fluorescence emission and scattered excitation light can propagate through the chip as wave guides. This is especially the case for *e.g.* POC systems which will require multi-channel microfluidic chips.[189] The propagated light can interfere with the detection of the fluorophores in the adjacent channels and chambers and/or cause photobleaching of the adjacent fluorescent samples. This crosstalk complicates the on-chip detection of fluorescently-labelled reporters primarily through an unpredictable fluctuation in the intensity of fluorescence.[189]

2.7 Functional μTAS

The driving force behind the development of microfabricated devices is the commercialization of microfluidic technology with its enormous potential of large-scale applications. A market analysis from 2005 stated that application of microfluidics in the life sciences had a global market of around 500 million euros, which could rise to about 1.4 billion euros by 2008.[190] Research has been concentrated on those areas that have the highest potential for short term commercial success. The most interesting applications of microfluidics chips and μTAS devices are those that would demand large volumes of microchips to low prices, such as public health monitoring, environmental monitoring, and for use in the medical systems of developing countries.[15, 81]

In the past decade, biology has quickly advanced from a state of mostly manual labour to early stages of automation. However, very few academic laboratories or companies use microfluidic devices for routine analyses, the macroscopic robots are still the choice for most when it comes to automatization.[191] One reason for this could be lack of interaction between technology developers within μTAS and the technology users. So far, integrated microfluidic systems lag behind the possibilities offered by robots and conventional fluid-handling tools when it comes to sophistication and parallelism. Most likely, it is only when the integrated microfluidic chips surpasses robotic capabilities and cost that widespread adoption of the microfluidic chips will be used in academic and industrial settings. Developing microfluidic devices that rival current robotic systems will require technology platforms that are capable of manipulating much smaller volumes while performing more complex tasks with higher degree of parallelization and integration.

Simulation tools are now used to make researchers able to rapidly determine how changes of design will affect chip performance, thereby reducing the number of prototyping iterations. However, the transition from simple microfluidic components to highly integrated systems is difficult as microsystems contain a network of microfluidic components that are linked in a more or less complicated manner. Most individual microfluidic components are often of little use unless they can be integrated in a functional system. It is highly desirable to decrease the reliance on external equipment, in order to achieve a higher degree of portability and hence fully realize the advantages of μTAS technology. However, the integration of microfluidic components, electrical components and optical components makes the system more complex and more prone to error unless all parts of the system are made robust. For instance, the functionality of microfluidic valves and pumps has shown to be unreliable. If only one part of the system not succeeds, the whole analysis will fail.

There are still several challenges related to integration of microfluidic components. Sample preparation is less developed than both separation and detection on microscale. In order to reach the goal of total integration, further integration of sample collection and sample preparation will be necessary to improve the interface between the microfluidic device and the surrounding macro environment, and to minimize potential cross-contamination. In addition, sealing and packaging represents a critical step in the production of microsystems. The available technology for efficient pumping, valving and on-chip reagent storage is limited as well.[81] Examples of integrated microfluidic systems can be found elsewhere.[69, 116, 192, 193, 194]

3 Summary of papers

Paper I

This article describes the first step in a project designed to downscale the nucleic acid sequence-based amplification (NASBA) reaction. The results present in the paper shows that it was possible to accomplish NASBA of artificial oligonucleotides in detection volumes of 10 nl and 50 nl. Reaction chambers operating with 10 nl as well as 50 nl were obtained using silicon-glass microchips. This is a reduction of the conventional reaction volume by a factor of 2000 and 400, respectively. A custom-made instrument with heat regulation and fluorescent detection was developed as well. NASBA is a well established method for diagnostic analysis, and the results from this work show the possibility of developing a LOC concept for the NASBA technology.

Paper II

In this work further development towards performing NASBA in nanoliter volumes is presented. It is described how it is possible to test a sample which is distributed automatically by capillary forces into 12 parallel and identical reaction chambers. The detection volume is 80 nl. Furthermore, the chip material was changed from silicon and glass to COC. The results from the experiments show that the detection limits of the artificial samples as well as the cell line samples are the same for cancer markers in nanoliter volumes as for conventional reaction volumes of 20 µl. A custom-made instrument was manufactured to increase light intensity, reduce component cost, and to integrate automatic actuation and optical positioning. The results obtained clearly substantiates that it might be possible to develop a LOC concept for the NASBA technology.

Paper III

The making of a novel non-contact pumping mechanism which enabled metering, isolation and movement of nanoliter sized sample plugs in parallel reaction channels is presented in this

manuscript. The mechanism was based on flexible COC membranes integrated on the microchip, combined with pins for actuation in the surrounding custom-made instrument. The COC chips with integrated pumps were able to simultaneously move parallel sample plugs along the reaction channels in four different steps. As the integrated pumps were designed to be used for NASBA, all the tests were performed at temperatures of 39˚C, 41˚C and 65˚C. The experiments revealed that the accuracy of the pump was highly dependent on the evaporation of sample and deformation of the COC membranes. The novel concept of this non-contact pumping mechanism shows potential as actuation mechanism for LOC devices. The advantages of these non-contact pumps are less risk for cross-contamination, between the separate reaction channels on the chip as well as between chips since no parts of the instrument are in contact with the sample. In addition, the integrated pumps membranes are low-cost which is essential for the production of disposable chips.

Paper IV

This manuscript presents experiments done both on macroscale and microscale, approaching a microchip in which all reagents are integrated. The goal was to apply the nucleic acids sample at the inlet of the microchip so that the NASBA procedure would automatically be performed on chip. Various methods for fabrication of microchips were investigated with regards to surface roughness and background fluorescence. Coating of the surfaces was required for amplification, as the native hydrophobic COC surfaces adsorbed proteins. A confocal microscope was used to determine the time for the dried mastermix and enzymes to dissolve. Protectants (trehalose, PEG, BSA and PVP) were added to the enzyme solution in order to protect their three-dimensional structures during drying and storage. On macroscale, successful rehydration and amplification were obtained for both dried mastermix and dried enzymes using HPV 16 oligonucleotides as well as CaSki cell lines as sample. However, only the dried enzymes gave successfully amplification of HPV 16 oligonucleotides on the COC chips. No amplification was observed for the dried mastermix on chip, and therefore this needs to be investigated further. It is suggested that the sequence in which the reagents were added to the microchip was of importance. Thus, the results of this work give some guidelines towards the development of a self-contained NASBA chip with regards to design, fabrication, surface modification, and amplification performance.

4 Results and discussion

The microchips developed in this work are general technology platforms that can be adapted to perform any clinical analysis which requires amplification of RNA or ssDNA. The objective of the present work was to develop a microchip for amplification and detection of mRNA by employing the real-time NASBA technology. Detection of HPV type 16 was used as a model target employing artificial HPV 16 oligonucleotides, SiHa cell lines and CaSki cell lines to demonstrate the functionality of the microchips.

Microfluidic technology focuses on picoliter, nanoliter and microliter fluid volumes and has allowed detection of single molecules at volumes in the picoliter range.[185, 195, 196] The reduction of reaction volumes lower the cost of analysis as the consumption of expensive reagents is reduced. However, these small volumes are not always suitable for realistic POC diagnostic applications. In most cases, the clinical samples contain low concentrations of the analyte of interest. In order to make reliable and robust μTAS applications, the sample volume, as well as the reaction volume should be large enough so that stochastic sampling effects will not be an important factor. On the other hand, if the sample volume required for detection becomes too large, microsystem technology will not be the best solution, as this will defeat the advantages of the approach.

The present work shows for the first time successful real-time amplification and detection employing NASBA in a microsystem format using a custom-made instrument. The NASBA reaction has been downscaled to the nanoliter level on silicon-glass chips, as well as on COC chips. The silicon-glass chips described in **Paper I** contained reaction chambers of 10 nl and 50 nl, which was a reduction of the conventional NASBA reaction volume by a factor of 2000 and 400, respectively. The 50 nl reaction, however, showed the same progress as in the conventional polypropylene tubes, while the 10 nl amplification curve had a different progress towards earlier signal increase. Several factors such as concentration variations and more efficient heat transfer of the silicon chip in comparison to the polypropylene tubes can contribute to the results obtained for the 10 nl reaction chambers. It is not possible to directly compare the results obtained for the 10 nl and 50 nl reaction chambers, as neither the target nor the concentrations of the sample are the same. To our knowledge, 10 nl is the smallest

detection volume known for NASBA. However, in comparison, the minimum reported size of reaction volumes for PCR and RT-PCR are 39.5 pl[197] and 450 pl[72], respectively.

While the silicon microchip only recorded fluorescent amplification signals of one reaction chamber, the COC microchips distributed the sample into 10 parallel reaction channels, with detection volumes of 80 nl, for simultaneous amplification and detection (**Paper II**). Dividing the sample to increase the number of simultaneous amplifications reactions are beneficial for diagnosis, as many diseases require identification of multiple targets. A second custom-made optical detection system was produced, in order to automatically detect the 10 parallel amplification reactions on the microchip. The NASBA results of the HPV 16 oligonucleotides and the SiHa cell line showed that all parameters (TTP, average slope, number of positive reactions) display the same trend for the microsystems as for the conventional methods, except for the fluorescence level ratios of the amplification reactions. As this ratio is almost constant for the microchip experiments, it decreases with sample concentrations for the conventional experiments. The fluorescence level is determined by the concentration of the molecular beacons in the reaction mixture. If the amplification reaches full reactant consumption, the final fluorescence should theoretically be independent of sample concentration, but reached at different times. However, dilution series of both artificial HPV 16 oligonucleotides and SiHa cell lines showed that the detection limits for the microchips were comparable to those obtained for the conventional routine-based laboratory-systems.

Further experiments with a silicon microchip containing detection volumes from 100 nl to 600 nl showed that the detection volume of 500 nl were most advantageous for artificial HPV 16 oligonucleotides, due to the relative increase in fluorescence signal (**Paper IV**). The reaction volume of the microchips was adjusted from 10 nl, 50 nl and 80 nl to 500 nl as the focus changed from cost of reagents on chip to robustness and reliability of the diagnostic application. The dilution series of HPV 16 oligonucleotides and the SiHa cell lines (**Paper II)** revealed that the limit of detection (LOD) of the conventional system and the 80 nl detection volume of the microchip was the same, 10^{-6} µM and 20 cells/µl, respectively. A SiHa cell may contain only 2 copies of the DNA virus but the number of mRNA transcripts in each cell is not known.[198] It was possible to detect even lower concentrations in both systems, but the results were inconsistent, most likely due to sampling effects. However, the custom-made instrument

was able to detect fewer molecules than the conventional system. It might be possible to obtain consistent amplification at lower concentrations than within the 80 nl microchips if the detection volume is increased to 500 nl. However, this was not tested in the present work.

For a conventional system employing 20 µl reaction volumes, it has been reported that as few as 50 copies of *in vitro* RNA/reaction have been detected in the case of NASBA, using molecular beacon.[199] However, the LOD is dependent on the target and, the LOD can vary from assay to assay due to the characteristics of the primers, the molecular beacon, type of sample material, the concentrations of the reagents within the reaction mixture, surface chemistry, heat transfer, and the quality of the detection system. The LOD of an assay is therefore not only limited by molecular restrictions of the reaction itself, but also of the surrounding system. One of the most sensitive assays, the micro PCR assay, was demonstrated by Lagally *et al.*[200] when a single molecule of DNA template was amplified in a glass device with reaction chambers of 280 nl. Marcus *et al.*[72] reported successful RT-PCR on chip for 72 parallel 450 pl reaction volumes for as few as 34 mRNA copies. Parallelization and simultaneous detection is important for high-throughput, and Leamon *et al.*[197] demonstrated simultaneous PCR amplification of up to 300 000 parallel reactions with individual reaction volumes of 39.5 pl. Belgrader and coworkers[201] reported that PCR amplification of "real" samples containing a starting template concentration as low as 5 *Erwinia herbicola* cells was feasible on silicon microchips.

On the other hand, Cepheid has developed a microfluidic device, GeneXpert®, for PCR and RT-PCR which implies detection volumes of 80 µl.[10] Its four-channel optics system is capable of dye detection with a limit of < 2 nM for FAM and Texas Red.[202] In comparison, the detection limit of HPV 16 oligonuclotides (**Paper II**) was consistent at 1 pM (10^{-6} µM). However, the GeneXpert can perform RNA isolation of pre-sliced and lysed cancer tissue samples, reverse transcription, and quantitative PCR in ~35 minutes.[10, 54] As microfabricated devices provide very rapid thermal energy transfer cycling, compared to conventional thermocyclers, it results in reduced analysis time on chip. However, as NASBA is isothermal, it can not benefit of increased heat transfer efficiency in the same manner as RT-PCR and PCR. Kopp et al.[114] demonstrated that a continuous-flow PCR chip had heating and cooling times less then 100 ms, which resulted in total reaction times of 90 seconds to 18.7 minutes for 20-cycle PCR amplifications (176 bp). But for NASBA the TTP will in most cases reveal if

the samples are positive long before the reaction is finished, unless the concentration of target is very low.

In most of the experiments performed in the presented work, which were not related to detection limits, a high concentration (0.1 μM) of HPV 16 oligonucleotides was added to the reaction mixture to ensure amplification. This corresponds to a copy number in the range of 10^8 - 10^{11} per detection volume between 10 nl and 20 μl. For a high viral load sample (*e.g.* HIV), the number of RNA copies entering the amplification reaction is in the order of 10^5 – 10^6.[32]

Two complementary mechanisms have been suggested for partial or complete inhibition of biological assays on microscale. Straight chemical inhibition[103, 106] or surface adsorption[105, 106] have been proposed, due to increased surface area-to-volume ratios in microchips. Of these, surface adsorption is regarded to be the most dominant for most assays. Bare silicon and silicon nitride have showed consistent inhibition of the PCR reaction, while silicon dioxide and polymer coatings have resulted in good amplifications.[103] The inhibition of the PCR reaction in glass-silicon chips is mainly caused by the adsorption of Taq polymerase to the walls of the chambers.[103, 105, 106, 203] Oda et al.[204] reported that DNA polymerase can also adsorb to metal. Protein adsorption is triggered by chemical and physical phenomena related to the surface materials and the surrounding medium. However, proteins tend to adsorb on to hydrophobic surfaces more than to hydrophilic surfaces. Both native silicon and COC surfaces are hydrophobic with regards to aqueous solutions. The net effect of polymerase adsorption can be counteracted by the addition of a titrated amount of competing protein BSA (dynamic passivation) or render the surface hydrophilic or neutral by static passivation. It is reported that the addition of BSA to counteract adsorption of Taq polymerase gives far better results than a simple five-fold increase of the Taq polymerase concentration.[106] Lou and coworkers[108, 109] have demonstrated that PEG and PVP are efficient for dynamic passivation of native and SiO_2-precoated silicon-glass chips in relation to PCR and ligase chain reaction (LCR) amplification. For many silicon-glass chips it was not enough to precoat the surface with SiO_2, as additional BSA was required to produce a positive reaction.[127] The NASBA reaction performed in conventional polypropylene tubes has been tested for biocompatibility of these reagents (**Paper IV**). However, this was tested in relation to stabilization of enzymes

during drying and has not yet been tested for dynamic surface passivation in microchannels. Panaro *et al.*[107] have described the surface effects and compatibility of PCR reactions on several common plastics, plastic tubing and disposable syringes. The results clearly show that some components of the material may inhibit the PCR reaction. The cause of the initial inhibition might be due to substances within the plastic that are released by the PCR reaction mixture or residual substances from the machining process.

Surfaces biocompatible with NASBA have been found to be a critical issue, in the same manner as for PCR. In microchips with native silicon surfaces (**Paper I**) and native COC surfaces (**Paper II**, **Paper IV**) it was not possible to amplify any target. Münchow *et al.*[117] showed that it was not possible to obtain PCR amplification in COC chips (Grade 5013) with reaction areas of native COC. However, PEG coated channels were reported to show good amplification. The NASBA reaction contains three different enzymes, in contrast to only one enzyme of PCR, and two enzymes of RT-PCR. As these enzymes consist of amino acids with a variety of side chains which can be either acidic, basic and have large variation in polarity, they respond differently towards a specific coating. In this work, it was not possible to label the three enzymes separately for adsorption measurements. Thus, the fluorescently labelled IgG mouse antibody was used as a model system. Adsorption measurements indicated clearly that fluorescently labelled mouse IgG bound unspecific to the hydrophobic native COC surfaces of the chip performing NASBA, while antibodies were rejected on the PEG coated COC surfaces (**Paper IV**). The amplification results obtained indicate that the NASBA enzymes adsorbed to native COC surfaces, as no amplification could be observed.

Successful amplification was obtained in silicon microchips modified with both SigmaCote™ and SiO_2 (**Paper I**, **Paper IV**). For silicon-glass chips, silanization compounds[103] and SiO_2 layers[9, 103, 104, 106] are the most commonly used coating types. For the COC chips, PEtOx-BP, PDMAA-BP and PEG were tested (**Paper IV**). Of the coatings tested for the COC microchips, it was the surfaces modified with PEG that showed the best biocompatibility with regards to NASBA. PEG is commonly considered the most effective polymer for protein resistant surfaces, because of its unique solution properties and its molecular conformation in aqueous solution.[121] However, the roughness of the surface to be coated can play a significant role for the coverage of coating on the surfaces. Surface

roughness can therefore contribute to a heterogeneous coating by leaving specific areas without coating. These uncoated areas are likely to adsorb reagents from the reaction solution.

In contrast to enzymes, DNA is not adsorbed to the walls in noticeable amounts.[106] However, depending on the pH, DNA can easily adsorb to glass and SiO_2. At pH 7.0, glass is negatively charged. When these surfaces are negatively charged, adsorption of divalent cations may occur. Due to the double layers thus formed, and because of the negative charge of DNA, it can be adsorbed on these surfaces.[205] Adsorption of oligonucleotides have not been tested in the present work, as this was regarded less important than protein adsorption.

In order to integrate the NASBA reagents on chip, a thorough evaluation of the reagents to be spotted and dried was needed. Because of the limited number of microchips available, it was necessary to map the most critical parameters on macroscale before transfer of knowledge to microscale (**Paper IV**). The DMSO and sorbitol enclosed in the standard final NASBA reaction mixture did not dry easily. Hence, it was found necessary to apply these compounds to the oligonucleotides or the sample of extracted nucleic acids before applied to the amplification chip. The standard NASBA reagents consist of the two main solutions, mastermix and enzymes in addition to the sample. The mastermix and enzymes were only stable, with subsequent successful amplification when spotted and dried separately. In a similar preliminary experiment for integration of PCR reagents on a chip, Weigl *et al.*[206] were able to dry and rehydrate the full PCR mixes in 96-well plates containing mastermix, and enzymes with varying concentration of trehalose. In this work, the protectants PEG and trehalose were also shown to be essential for recovery of enzyme activity in the NASBA reaction after drying on macroscale. The protectants were tested on both oligonucleotides and CaSki cell lines, as previous results (data not shown) have revealed that oligonucleotides are more easily amplified than both cell line samples and clinical samples. However, when testing PVP and BSA, as well as different combinations of the protectants, only amplification of the oligonucleotides could be observed.

The quality of the assay depends on how the sample material is collected and how the sample material is handled and processed before analysis takes place. The extracts of nucleic acid of the cell line contain a pool of genomic DNA, non-coding and coding RNA of various structures. Complex intramolecular structures in addition to a large spectrum of different kinds

of molecules complicate the process of hybridization of primers and binding of enzymes to the targets. It is important that everything except the nucleic acids is removed through the extraction. In the case of oligonucleotide samples, all the molecules are targets. However, it is important to purify the target, as impurities produced during synthetization may be inhibiting.

In order to prepare for the temperature control of the chip, it was also shown that the dried enzymes were stable at 65˚C for up to 2 hours, while enzymes in solution were inactivated after only 3 minutes at 65˚C (**Paper IV**). The mastermix and sample are normally heated to 65˚C for 3 minutes prior to addition of enzymes. This procedure is performed to break down the secondary structures of the nucleic acids, and to ease the hybridization of primers to the target of interest. These experiments showed that the enzymes are more temperature stable when dry, than when in solution. This was expected, as drying of reagents are normally used for long-term storage.[142]

It was necessary to render both the mastermix and enzymes fluorescent prior to drying in order to evaluate the rehydration and diffusion rates of these reagents into the sample on chip (**Paper IV**). Molecular beacons without quenchers were dried with the mastermix to record the rehydration rate of the mastermix. These molecules were amongst the largest of the compounds in the mastermix and thus, have the slowest diffusion. The diffusion depends on temperature, viscosity, size and shape of the molecule. The modified molecular beacon is slightly smaller than the regular molecular beacon, although not much, and this difference would most likely not affect the diffusion rate significantly. However, these experiments were performed at room temperature, while on chip rehydration will occur at 65˚C. Higher temperatures increase the diffusion coefficient, which again leads to faster diffusion times. The time for diffusion of the modified molecular beacons in the mastermix was ~60 seconds, which roughly corresponds to the calculated time needed to obtain a homogeneous suspension. Diffusion measurements of the molecular beacons without quencher in the mastermix implied that the dissolution process was diffusion-limited.

The fluorescently labelled mouse IgG (150 kDa) dried with the enzymes, modelled the diffusion rate of the largest NASBA enzyme (AMV-RT, 160 kDa). The IgG were assumed to be smaller and with a different shape compared to the globular AMV-RT enzyme. The rehydration/diffusion processes for the dried enzymes was significantly slower than for the dried mastermix. A homogeneous suspension was observed after approximately 10 minutes at

room temperature. The estimated time for diffusion for the IgG was ~3 minutes. However, the estimate is based on a model for spherical molecules. As the real shape and size was not known, parameters could contribute to the deviation between observation and estimate. During drying, films which are difficult to dissolve may be created on the surface of the reagents. It is not unlikely that this was the case, as similar experiments showed that the dried reagents were not dissolved at all. Hence, in this case, the measurements implicated that the rehydration process of the enzymes was slower than their diffusion into the bulk liquid. The experiment was performed at room temperature, but ought to have been performed at 41˚C as it will eventually be performed on the chip.

Only dried enzymes with 0.05% PEG protectant have been successfully amplified on chip (**Paper IV**). The TTP was ~15 minutes, showing that the rehydration rate of the enzymes is sufficient to obtain amplification. The fluorescent ratio is about 6 times, revealing that the activity of the dried enzymes was recovered upon rehydration. Puckett *et al.*[153] and Garcia *et al.*[152] have shown the ability to dry biological reagents, and later to reconstitute them, on a microfluidic platform without inhibition of the assay.

Successful amplification of the rehydrated mastermix on the microchips employing NASBA still remains. The reason for this is not clear, as the dried mastermix worked on macroscale. The order in which the reagents were applied to the chip could be one explanation. On chip, the rehydration of the mastermix was accomplished by applying a mixture of the enzymes, DMSO/sorbitol and oligo sample. The dried and highly concentrated mastermix deposited on the chip could influence the activity of the enzymes in the mixture entering the chip. For instance, the T7 RNA polymerase is extremely sensitive to salt concentrations. It is obvious that the order in which the reagents has to be applied must be investigated more thoroughly.

Paper IV indicates that the ratio of concentration between the mastermix and enzyme reagents is of importance. Optimalization of the NASBA reagents on chip still remains to be tested. However, some work has been performed to optimize PCR on silicon-glass chips, as well as polymeric devices. [69, 105] As many of the reagents are the same for both reactions, it might be advantageous to test similar conditions for NASBA. Variations on the composition of reagents are possible, but the concentration of some components is critical. In general, the

enzyme concentrations for PCR were increased by a factor of 1.5-4 to produce yields equivalent to the tube-based assays.[69, 105] Increased amounts of BSA are often applied, due to its ability to provide a dynamic coating. In addition, BSA stabilizes labile enzymes, and it can even be required for enzyme activity. As NASBA requires BSA for enzyme activity, it is likely that increased concentrations of BSA are required for optimal results. Furthermore, a significant increase in enzyme concentration would require experimental titration of Mg^{2+} ions, as it is an essential co-factor in many enzymes, including RNase H and DNA polymerases. However, high concentrations of Mg^{2+} ions could have a net influence on the pH, thus triggering a cascade of experimental titrations for other reagents (*e.g.* KCl). The Mg^{2+} ion concentration required for optimal amplification may depend on the specific set of primers, and also for the template used, as it is bound by oligonucleotides and dNTPs. Salt-dependent electrostatic effects are major factors in determining the stability, structure, reactivity and binding behaviour of all nucleic acids. The cations stabilize the base pairs in the following order: $Ca^{2+} > Mg^{2+} >> K^{+} > Na^{+}$, showing that both the charge and the ionic radius affect the stabilization of the duplex.[207] The optimal KCl concentration in the reaction mixture can vary for different targets and primer combinations. The salt concentration alters the secondary structure of the nucleic acids. An optimal salt concentration for the NASBA amplification is obtained when the secondary structure of the target is minimal, to allow good annealing with the primers. Hence, the KCl concentration will also affect the hybridization kinetics of the individual primers and thereby increase the specificity of the primer annealing. If the concentration of molecular beacon probes is too low, it will limit the detection of fluorescence. However, if it is too high, it would disturb and inhibit further RNA amplification.[30] Optimal concentration of DMSO is template dependent. For NASBA applications, the DMSO concentration should normally not exceed 16-17% in the final mixture, as it may inhibit the reaction. If the concentration is too low non-specific amplification may occur.[208] Addition of DMSO improves the specificity, efficiency and yield of the amplification reaction. DMSO and sorbitol have been reported to lower the melting temperature of nucleic acids duplexes.[209] Additionally, DMSO has been shown to accelerate strand renaturation and is believed to provide increased thermal stability for nucleic acids, thus preventing depurination. DMSO is an agent that disrupts mismatching of nucleotide pairs.

Design and fabrication methods of the microchips used were found to be crucial for chip performance. Rough surfaces do not only create background noise for the optical measurements, but also contribute to generation of bubbles and problems related to manipulation of the sample within the network of channels and chambers. The silicon microchips were manufactured with optically smooth surfaces, which are defined as having a surface roughness of less than 1/10 of the excitation wavelength (**Paper I**). However, low surface roughness was difficult to obtain for the COC microchips. Of the fabrication methods evaluated, it was the injection moulded chips which showed the smoothest surfaces closely followed by the hot embossed chips (**Paper IV**). Milling and laser ablation typically used for rapid prototyping, produced the roughest surfaces. For the milled microchips, it became clear that the rough surfaces also contributed to loss of liquid as the plugs were transported through the channels. Additionally, it was observed that cross-sectional constrictions in the channels resulted in loss of liquid when the liquid plugs were moved through these structures. A self-contained microchip for performing NASBA will include an increased number of geometrical constrictions. It is likely that some liquid will be lost during transport through the network, and therefore increasing the reaction volume to 500 nl will ensure that sufficient sample is left for detection.

Liquid loss was also experienced due to evaporation, which increased at elevated temperatures. In order to minimize the evaporation within the system, it was important to close the chips and to reduce the dead volume and the diffusion lengths. For the non-contact pump mechanism (**Paper III),** it was shown that the total sample volume was reduced after the sample plugs were moved from the first reaction site to the third reaction site. The volume lost was 3.3 nl after 2 minutes at 65˚C and 4.1 nl after 7 minutes at 41˚C. In addition, the evaporation increased the partial vapour pressures in the upstream closed side of the channel and thus expanded its volume. In some cases, this resulted in a movement of the sample plugs towards the second position. The inlet hole and the air venting at the waste chamber was not sealed during these experiments. If microreactors are open to the surrounding environment, the amplification volume will shrink. Thermal gradients might also induce bubble movement, and the evaporation itself could cause liquid pumping.[148] Consequently, reaction chambers should be closed. Physical valves can be used to keep in place the reagents and prevent migration and evaporation.[69, 116] Mineral oil has been commonly used to seal off reactions chambers to avoid

evaporation on microchips as well.[204, 210, 211] However, Panaro et al.[107] found that mineral oil can inhibit PCR either completely or partly. The reason for this could be that the compounds which are dissolved in oil will migrate into the oil and no longer be available for the reaction. For open PCR reactors, significant bubble formation has been observed.[212] The thermocycling of the PCR reaction reaches maximum temperatures of ~95˚C, thus close to the boiling temperature of water. For NASBA, which is isothermal at 41˚C, one would expect less bubble formation. However, bubble formation was a large problem for chips performing NASBA when the surface roughness of the reaction chambers was large, even though the chips were sealed (**Paper IV**). For the silicon and COC chips in **Paper I** and **Paper II**, bubble formation was not found to be a significant problem. For the chips with large surface roughness, bubbles were mostly trapped during filling and tended to stick to the channel surface and disturbed the reagent flow. In some cases, these bubbles expelled the reagents from the reaction chambers, thereby reducing the amplification efficiency. Bubbles trapped inside a microchamber may undergo expansion, contraction, and relocation inside the microfluidic network, depending on the temperature regulation of the microsystem.[213] A possible remedy to minimize or eliminate bubble formation is to design reaction chambers which avoid the trapping of air bubbles, and to pressurize the reaction chambers by closing them.[213]

The sealing between the chip itself and the top layer enclosing the channel system was of importance, due to the possibility of leakage, which in turn can enhance evaporation. The silicon-glass chips used in the experiments described in **Paper I**, were sealed by the use of anodic bonding. This technique makes strong sealing between the chip and the top layer, but requires high temperatures (~400˚C) and voltages. Therefore, this technique would cause a problem in cases with deposited biological reagents on the chips. For the COC chips described in **Paper II** and **Paper III**, solvent welding was used. However, brittle membranes which easily cracked up were a problem with this sealing method. In addition, deposited reagents might be exposed to the solvent used for the welding. For this reason solvent welding was not the best alternative. Adhesive pressure sensitive tapes were used to seal the silicon and COC chips used in **Paper IV**. The adhesive on the tape did not seem to interfere with the amplification reaction. But due to hydrophobicity of the adhesive, it became difficult to fill the reaction chambers designed for spotting. Huge problems related to these tapes had to do with leakage of sample if the sealing surface of the chips was covered with PEG coating. However,

this adhesive tape would not be an option for the pumping mechanism presented in **Paper III**, as the tape would have glued itself to the bottom of the actuation chambers.

A novel non-contact pumping mechanism based on on-chip flexible COC membranes combined with actuation pins in the surrounding instrument was tested and evaluated (**Paper III**). The mechanism enabled metering, isolation and movement of nanoliter sized sample plugs in parallel reaction channels. The COC chips with integrated pumps were able to simultaneously move parallel sample plugs along the reaction channels in four different positions. Each reaction channel contained a set of 4 actuation chambers in order to obtain metering, isolation and movement of the sample plug into the detection area. The pump accuracy depended on the evaporation and deformation of the COC membranes used. Successful amplification of artificial HPV 16 oligonucleotides plugs of approximately 96 nl (detection volume of 80 nl) was obtained on premixed NASBA reaction mixture. However, these amplification results are not discussed in **Paper III**. In most of the measurements, the sample plugs were relocated from the detection area. Hence, it was no longer possible for the optical detection system to record the fluorescent signal of the amplification. This was the reason for not using the non-contact pumping mechanism for the experiments in **Paper II**. Either geometrical constrictions or valves should have been implemented within the reaction channels in order to fix the sample during measurement.

A syringe pump in combination with capillary forces was tested on the COC chips in **Paper IV**. As mentioned previously, the design of the microfluidic network affected the filling and encapsulation of bubbles, actuation and movement of the liquid plugs to the reaction chambers. Large reaction chambers were required in order to be able to perform spotting of reagents. Otherwise the reagents would spread out through the whole microfluidic network if the spotted liquid came in contact with the walls. The sample containing DMSO and sorbitol for rehydration of the mastermix showed good wetting properties of the PEG coated surfaces. The combination of the DMSO and sorbitol solution and the hydrophilic surface induced the liquid to start creeping trough out the microfluidic channels along the channel corners. In this case, the sample plug applied to the chip did not obtain static equilibrium of the meniscus, due to the high wetting properties of the sample plug on this PEG coated surface. The instability of the liquid will drive the wetting along the edges up to the very end of the channel system, as

long as sufficient liquid is provided in the system.[84, 213] This creeping effect was able to drain the reaction chambers during metering and movement. A capillary stop valve was introduced in the channel by spotting a hydrophobic patch (Teflon AF). The capillary valve stopped the creeping liquid behaviour and pressure differences of ~1000 Pa were required to overcome the strength of the valve in order to move the liquid. However, such capillary stop valves can produce possible gating inefficiencies, when liquids with low surface tension are used.[214]

Two custom-made optical detection instruments were developed in order to measure the NASBA reaction in nanoliter volumes on a microchip (**Paper I**, **Paper II**). For the second instrument, it was possible to obtain 56 times the excitation light intensity on the reaction channels compared to the first instrument. The increase is mainly due to a brighter light emitting diode (LED). The silicon chips had an optically smooth, reflecting and non-fluorescent surface, where the fluorescent light was spread uniformly into a hemisphere, assuming that the channel walls were 100% reflecting (**Paper I**). The detection optics would collect 8% of the excited fluorescent light. In the case of a transmitting chamber wall (polymer materials) with an opening angle of the collecting angle of ± 26°, only 5% of the excited fluorescent light was collected, as the fluorescent light was spread into a sphere instead of into a hemisphere (**Paper II**, **Paper IV**). In addition, polymer materials usually display autofluorescence. However, polymer materials may be bleached when exposed to light for longer periods of time.[96] Further, the surfaces of polymer materials is rougher in the range of 0.03 µm to 16.6 µm (**Paper IV**), while the surface roughness of the silicon chips were in the order of 1/10 of the excitation wavelength used (**Paper I**). When the surface roughness is large, the excitation and emission lights in the measurements are scattered and the optical pathway is disturbed, which may lead to unreliable measurements. However, the surface roughness varies considerably, depending on the chip material and fabrication method used. Injection moulded, hot embossed and micromachined silicon microchips usually offer lower surface roughness compared to milled and laser ablated microchips. Therefore, these rough surfaces will be less suitable as optical elements.

The solvent background signal can be drastically reduced by minimizing the optical detection volume, since the signal from a single molecule is dependent on probe volume dimensions, whilst the background scales proportionally with the probe volume.[46] For very

low concentrations of target, an increased detection volume is generally undesirable, since the background interferences increase. The various components of the NASBA mixture, either individually or in combination, can contribute to an increased value of background fluorescence, in addition to that produced from leakage from the molecular beacon. This has not been evaluated in this work. However, Obeid et al.[71] found that the combination of primers and Triton X-100 in their PCR reaction mixture contributed to the high background fluorescence, although, neither primers nor Triton X-100 showed high background when studied separately.

It is often the background noise which limits the sensitivity of an assay. The signal-to-noise ratio is the power ratio between the signal and the background noise and it should be as large as possible in order to obtain the good sensitivity. In addition to reduction of the background fluorescence of the chip material and the reagents in use, the LED and the photomultiplier tube (PMT) components of the instruments depend on temperature. Hence, the fluorescence measurements will have a small variation on a day to day basis. For a successful NASBA reaction, the signal to noise ratio was typically in the order of 1000 for the COC chips.

The first instrument which was used required manual positioning of the silicon chips (**Paper I**). It was difficult to place the microchip in the exact same position every time. This resulted in discrepancies between samples, negative controls as well as the starting point of amplified targets. In addition, variations in concentrations can explain the level of fluorescence. The error introduces a relative shift in detector readout and does not affect the amplification process itself. The results show a distinct difference between negative controls and the amplification curve. The start signal of both the 10 nl and the 50 nl reaction chambers were approximately 2-fold compared to the background signal of empty silicon chips. The fluorescence signal was amplified by a factor of 2 for both chips during the reaction, while the conventional amplification showed an increase of ~7 times. However, this is expected, as baseline offset will become more significant as the reaction volume decreases, and it will dominate at low signal levels. The ratio between the start signal and the background signal in the second instrument and the chips with 10 parallel reaction channels were in the order of 3 (**Paper II**). No crosstalk was detected between the parallel reaction channels. The experiments showed that the ratio between the final fluorescence level and the initial fluorescence level for

the amplification on the microchips was nearly constant for all sample concentrations, while it decreased with sample concentration for the conventional experiments. The overall lower ratio obtained in the microchips could be explained by the enlarged background signal, caused by autofluorescent COC and light scattering from imperfect polymer surfaces. The background signal (**Paper IV**) varies quite a lot also due to the material and backgrounds. Also, the results described in **Paper IV**, illustrates that the field of view is critical. When optimizing the detection volumes in the silicon chips, volumes from 100 nl to 600 nl were used. The optical view of the instrument was adjusted to be able to detect the whole 600 nl chamber. As a result, the signal from the experiment using 100 nl is most likely lost because of the background signal, and the straight line is due to this and not to unsuccessful amplification. It has been possible to perform NASBA at even lower detection volumes. The signal from the 200 nl detection volume is also very low. However, a certain increase is observed. The results presented show that it would be of advantage to increase the detection volume to 500 nl as the increase in signal from 500 nl to 600 nl was not significant.

5 Concluding remarks and future perspectives

To our knowledge, this work presents the very first work employing NASBA in a microchip format at the nanoliter scale. The conventional NASBA reaction was at first reduced by a factor of 2000. However, later the reaction volume on chip was in subsequent experiments increased from 10 nl to 500 nl in order to make more robust and reliable diagnostic applications. The detection limits were found to be comparable to those obtained for experiments performed in conventional routine-based laboratory systems for both oligonucleotides and cell lines. In addition, identification of different targets in one and the same clinical sample by simultaneous detection in multi-parallel reaction chambers is most likely possible and indicates that the microchip and its detection system has a potential for diagnostic use in a POC setting.

Several aspects of the development of a self-contained microchip for NASBA have been tested. The present work provides useful guidelines towards the development of a complete automatic chip performing NASBA. Still, a number of important issues remain to be studied before satisfactory results can be obtained. However, integration of self-contained sample preparation chips along with amplification and detection chips would constitute a fully automatic, laboratory independent diagnostic system. Further, the integrated diagnostic system have to validated with regard to sensitivity, risk of cross-contamination, robustness and the reliability of the system using clinical samples.

This work will be continued through two projects; MicroActive (IST-2005-017319)[215] and SmartHEALTH (FP6-2004-IST-NMP-2-016817)[216] within the EU's 6th IST Framework Programme.

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