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PAPER

A phaseguided passive batch microfluidic mixing chamber for isothermal amplification†

Sydney Hakenberg,^{*a} Matthias Hügler,^a Manfred Weidmann,^b Frank Hufert,^b Gregory Dame^a and Gerald A. Urban^a

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With a view to developing a rapid pathogen detection system utilizing isothermal nucleic acid amplification, the necessary micro-mixing step is innovatively implemented on a chip. Passive laminar flow mixing of two 6.5 μ l batches differing in viscosity is performed within a microfluidic chamber. This is achieved with a novel chip space-saving phaseguide design which allows, for the first time, the complete integration of a passive mixing structure into a target chamber. Sequential filling of batches prior to mixing is demonstrated. Simulation predicts a reduction of diffusive mixing time from hours down to one minute. A simple and low-cost fabrication method is used which combines dry film resist technology and direct wafer bonding. Finally, an isothermal nucleic acid detection assay is successfully implemented where fluorescence results are measured directly from the chip after a one minute mixing sequence. In combination with our previous work, this opens up the way towards a fully integrated pathogen detection system in a lab-on-a-chip format.

1. Introduction

Lab-on-a-chip solutions have been recognised as an opportunity to bring accurate and sensitive diagnostic tests to the point-of-care (POC).¹ For a complete integration of the latest innovative assays, e.g. RPA,² unit operations such as micro-mixing need to be implemented on chip. A simple but efficient passive micromixer is the choice for many applications in chemical and biochemical analyses.³

In microfluidics low Reynolds numbers are common due to the small dimensions and therefore the fluidics are dominated by laminar flow.⁴ This is making mixing significantly more difficult than in macrofluidics, because turbulences are not occurring in laminar flow characteristics and stirrers in the usual sense are not available.⁵ Therefore, the only passive mixing principle is to shorten diffusion time by increasing the contact areas between the liquid species and to shorten the diffusion length. In the literature there are several methods published to achieve this. Among the most common ones⁶ are T-mixers,⁷ Y-conjunctions, split-and-recombine concepts,⁸ hydrodynamic focusing,⁹ flow obstacles within microchannels and meander-like or zig-zag channels. Active mixing principles can be very efficient and range

from pneumatic wall movement¹⁰ and acoustic actuation¹¹ to periodic injections,¹² but they usually require extra fabrication steps or additional peripheral actuation devices.

The large majority of micromixers are designed for continuous mixing, but frequently microsystems try to integrate biological or chemical assays where single components might be expensive and limited. Here, batch mixing would be beneficial and cost saving. Some of the introduced micromixers might achieve batch mixing in combination with a dosing concept, but only a few indicate their mixing methods as batch-mode^{13,14} compatible.

In microfluidics, so far all passive pressure driven mixing structures require additional space on the chip in order to achieve efficient mixing.^{5,15} In this novel approach, that space is successfully omitted by conjoining the mixing section and the destination chamber for the mixed species. Mixing structures aiming for efficiency and negligible chip space often require complex fabrication technologies.¹⁶ However, here a simple and cost saving fabrication method is used.¹⁷

A passive microfluidic batch mixing chip is presented which is based on the recently introduced phaseguide technology.¹⁸ Thus complete control over the filling and emptying of any type of microfluidic structures is given. This allows, for the first time, the direct integration of a mixing structure into a functional microfluidic chamber, such as the detection chamber of an assay. The applicability of this system is demonstrated with isothermal recombinase polymerase amplification² (RPA) on chip including its necessary mixing step. This detection assay, in combination with the ease of integration of phaseguided fluid handling, is promising with a view to a new generation of point of care diagnostic systems.

^aLaboratory for Sensors, Department for Microsystem Engineering (IMTEK), Albert-Ludwigs-University Freiburg, Georges-Köhler-Allee 103, 79110 Freiburg, Germany. E-mail: hakenberg@imtek.de; Fax: +49 (0)761 203-7262; Tel: +49 (0)761 203-7268

^bInstitute for Virology, University of Goettingen, Kreuzberggring 57, 37075 Goettingen, Germany

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2. Materials and methods

2.1 Chip fabrication

Fabrication methods using dry film resist technology with subsequent direct wafer bonding are described in detail elsewhere.¹⁷ This dry film resist is a 30 μm thick Ordyl SY330, which is laminated onto a Pyrex glass substrate. It is the base material for microfluidic structures and at the same time it also serves as the wafer bonding material. The first laminated layer forms the phaseguide design after a lithography step. Three more layers build the channels and chamber walls with the second lithography. After developing the Ordyl resist, a second Pyrex substrate with drilled holes for microfluidic inlets and outlets is directly bonded onto the structured wafer. This method results in 120 μm high microfluidic chambers and channels where the phaseguide microstructures make a quarter of the height. In the final step of the chip's production the wafers are diced.

2.2 Phaseguides

The phaseguide technology is based on pinning of the liquid front where the Concus-Finn criterion is fulfilled.¹⁹ This is achieved by topological Ordyl obstacles in the microfluidic chambers. Phaseguide theory and detailed principles have been described previously by Vulto *et al.*¹⁸ In Fig. 1 a meniscus pinning is shown as an overflow pressure barrier. The liquid front is aligned along the phaseguide in the chip filling the whole segment completely before it jumps over to the next segment at a higher pressure. This basic phaseguide principle has been used earlier for a bubble free filling of functional microfluidic chambers.^{20,21} Here it is also used in the parallel filling chamber for subsequent batch filling and simultaneous emptying. On this mixing chip, it is shown for the first time that phaseguides are also capable of filling a microfluidic chamber in a more complex way, including defined meander-like paths. In Fig. 1 b it is indicated how phaseguides are used on this chip in the mixing/target chamber. In a single chamber two liquid flow columns are kept separated from each other by phaseguide meniscus pinning. Liquid flow is bounded by two phaseguides running parallel to its flow direction, thus leaving air as a barrier between two columns.

Fig. 1 Cross-sectional area (above) and topview sketch of the microfluidic chip (below). Liquid flow direction is indicated by arrows and cross-sectional planes by red dash-dotted lines. a) Meniscus pinning occurring at a topological obstacle before overflow. b) Two liquid flow columns are kept separated from each other by phaseguide meniscus pinning. As indicated by symbols, the flow direction of such separated columns can be different.

Fig. 2 Layout of the microfluidic mixing chip. Top right micrograph shows a microscopic enlargement of phaseguides and a chamber wall from a fabricated chip. The phaseguides encounter the chamber wall in a sharp angle for a defined overflow during the initial parallel filling.¹⁸

2.3 Chip layout and operation

The layout of the chip is illustrated in Fig. 2. The chip is inserted in a chip-holder which clamps a standard tube connector (*The Lee Company*) onto the outlet. A PTFE tubing is used and the contact area is sealed with an O-ring. The whole chip setup is actuated by a syringe pump.

First DI-water batches are coloured with methylene blue and neutral red for visualization. Successively the two liquids to be mixed are manually introduced into the inlets 1 and 2 respectively using a pipette. In this chip layout the two batches contain 6.5 μL each. Finally, the outlet is connected to a syringe pump set to a flow rate of 15 $\mu\text{L min}^{-1}$ which initiates the passive mixing process.

2.4 Recombinase polymerase amplification

RPA is an isothermal oligonucleotide amplification method operated at low, constant temperatures.² Therefore no thermal cycling device is needed for target molecule amplification and detection, which makes RPA an ideal candidate for miniaturized point of care diagnostics. A primer probe system similar to real time polymerase chain reaction is used. All reagents named in this section were purchased from *TwistDx*. While performing this reaction on chip, liquid 1 contains rehydration buffer, magnesium acetate and roughly 100 copies of positive control DNA. Liquid 2 contains a primer/probe mix and a lyophilisate containing all necessary enzymes dissolved in rehydration buffer. Thus mixing of the two liquids is mandatory for the reaction to start. The RPA signal is detected in the mixing/target chamber with a *Nikon Eclipse TE2000-U* inverted fluorescence microscope, while maintaining the chip temperature at 39 $^{\circ}\text{C}$ with a self-constructed chip holder. The centrepiece of this chipholder is a Peltier device and a controller. A custom-made copper chip fixture works as a heat conductor and as a microscope adapter.

3. Results

3.1 Mixing sequence

The fluidic mixing sequence on chip is shown in Fig. 3 a–f using coloured water. Phaseguides are forming continuous pinning

Fig. 3 Mixing sequence on chip demonstrated with coloured water. Chronological order is indicated by arrows from parallel filling towards mixed state. Video clips are available in the ESI.†

barriers thus allowing precise segmented and sequenced filling. In the parallel filling chamber, the fishbone-like phaseguide's design keeps the two liquid volumes side by side in an unmixed state. This parallel filling sequence is achieved by different phaseguide-wall angles within the chamber. Liquid overflow occurs at sharp angles rather than obtuse angles when pressure prevails over meniscus pinning.¹⁸ This behaviour is shown in Fig. 3 b where liquid overflow occurs at the blue liquid front while the red liquid front remains in position by meniscus pinning.

Under actuation the parallel filling chamber is emptied while the incoming air is drawn from the vent. Both liquids are transferred simultaneously and evenly due to the phaseguide segmented chamber and surface tension which provokes the minimization of the liquid surface area at the receding interface. This behaviour is shown in Fig. 3 b, d and e where both liquids are transferred with an even receding front. At the same time the liquids advance along the mixing structure within the destination chamber. Until this open chamber is half filled, the mixing behaviour is similar to a meander or zigzag laminar flow mixer.²² This is followed by additional outward artificial twisting of the liquid interfaces during further filling of the destination chamber from the interior. Once this chamber is filled completely the two liquids are in a mixed state within <1 min. As in any other laminar flow micromixer, the mixing principle is to shorten the diffusion length between the liquids. This phaseguided principle is also working for liquids clearly differing in viscosity, which is the case for the RPA reagents. Both liquids are transferred with an even receding front and at the same speed while advancing along the mixing structure.

3.2 Simulation of diffusive mixing

In order to optimize and to estimate the benefit from the mixing step the diffusion behaviour was simulated. *COMSOL Multiphysics* simulation results with real chip dimensions are shown in Fig. 4. The chosen diffusion coefficient was $3 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ which is

about the same range as water self-diffusion. The distribution of the two fluid concentrations are shown in Fig. 4 a after 1500 s. The upper picture shows the mixing chamber assuming the fluids are initially running side by side in the phaseguide paths until they are completely filled before diffusion starts. The bottom picture compares the chamber assuming ideal side by side filling and diffusive mixing only. Where a 0.5 value represents the perfectly mixed state and 1 and 0 the two unmixed fluids, the mixing chamber simulation ranges from 0.47 to 0.53. On the other hand, diffusive mixing results in a range from 0.05 to 0.95.

Fig. 4 b compares the area average value of concentration in mol m^{-3} of the two chambers as a function of time. It results in complete diffusive mixing within 1 min after the mixing step, while for parallel filling it would take more than one hour. This indicates that this batch mixing chip clearly assists the passive diffusive mixing in less than one minute.

3.3 RPA on chip

The mixing chip was tested using the RPA assay. Liquid 1 and 2 (section 2.4) clearly differed in viscosity, but this had no influence on the mixing sequence (section 3.1). The positive control measurement in Fig. 5 was prepared as described in section 2.4 as well as the negative control, but the DNA in liquid 1 was replaced by DI-water. Fluorescence remained more or less at the same level except for one artefact spike, which probably occurred due to interfering light near the measurement setup. The positive signal clearly shows an increase in fluorescence during the RPA reaction. This result clearly demonstrates the proof of principle and the sufficiency of the mixing chip for running an RPA assay. Due to the fabrication method (section 2.1), the liquid layer in the chip from which the signal was detected is 120 μm high. Increasing the height of this liquid layer will significantly increase the relative fluorescence difference between the two measurements. This can be achieved, for example, by a lateral measurement through the 12 mm detection

Fig. 4 COMSOL Multiphysics diffusion simulation results of two fluids with a diffusion coefficient of $3 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$. Chip dimensions are used. a) Graphic visualization of the diffusive mixing state after 1500 s: The top picture shows the mixing chamber given an ideal side by side filling with phaseguides. The bottom picture shows the same sized chamber given an ideal parallel filling. b) The area average values of concentration (mol m^{-3}) after phaseguided mixing and parallel filling are compared as a function of time.

Fig. 5 Fluorescence microscope RPA signal measurement from the mixing/target chamber. The positive control reaction shows an increase of fluorescence while the no template control remains about at the same fluorescent level which demonstrates that mixing efficiency is sufficient for this example of a biological assay.

chamber width, but that method isn't feasible with the currently used chip-holder. Hereby, the signal to noise ratio and the sensitivity can also be increased.

4. Conclusion

Mixing of two 6.5 μL batches within one minute was demonstrated and visualized with coloured water on a chip. For the first time passive microfluidic mixing was performed within one single chamber. The mixing efficiency benefit was estimated with simulation results. This chip reduces the diffusive mixing time on the microscale from hours to one minute for diffusion coefficients in the range of water self-diffusion. Furthermore, it was demonstrated that its mixing efficiency is also sufficient for an isothermal nucleic acid detection method. RPA is one of many possible applications of this mixing chip. Fundamentally

the mixing chip allows the introduction of two separate liquids next to each other in a very defined and reproducible way and to subsequently mix these two liquid species. This can be crucial in any microfluidic assay where it is necessary to introduce one or more components successively. Mixing of this type appears possible for liquids differing in viscosity, but the range of this difference still needs to be determined.

The combination of our on-chip electrophoretic RNA extraction module²¹ with this mixing chip could open the way towards a rapid and low cost pathogen detection system in lab-on-a-chip format.

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References

- 1 P. Yager, G. J. Domingo and J. Gerdes, *Annu. Rev. Biomed. Eng.*, 2008, **10**, 107–144.
- 2 O. Piepenburg, C. H. Williams, D. L. Stemple and N. A. Armes, *PLoS Biol.*, 2006, **4**(7)(e204), 1115–1121.
- 3 N.-T. Nguyen and W. Zhigang, *J. Micromech. Microeng.*, 2005, **15**, R1–R16.
- 4 P. Gravesen, J. Branebjerg and O. S. Jensen, *J. Micromech. Microeng.*, 1993, **3**, 168–182.
- 5 S. Hardt, K. Drese, V. Hessel and F. Schönfeld, *ASME Conference Proceedings*, 2004, **2004**, 45–55.
- 6 V. Hessel, H. Löwe and F. Schönfeld, *Chem. Eng. Sci.*, 2005, **60**, 2479–2501.
- 7 S. H. Wong, M. C. L. Ward and C. W. Wharton, *Sens. Actuators, B*, 2004, **100**, 359–379.
- 8 F. Schönfeld, V. Hessel and C. Hofmann, *Lab Chip*, 2004, **4**, 65–69.

- 9 T. T. Veenstra, T. S. J. Lammerink, M. C. Elwenspoek and A. v. d. Berg, *J. Micromech. Microeng.*, 1999, **9**, 199–202.
- 10 S.-K. Hsiung, C.-H. Lee, J.-L. Lin and G.-B. Lee, *J. Micromech. Microeng.*, 2007, **17**, 129–138.
- 11 Z. Yang, S. Matsumoto, H. Goto, M. Matsumoto and R. Maeda, *Sens. Actuators, A*, 2001, **93**, 266–272.
- 12 I. Glasgow and N. Aubry, *Lab Chip*, 2003, **3**, 114–120.
- 13 M. Grumann, A. Geipel, L. Riegger, R. Zengerle and J. Ducree, *Lab Chip*, 2005, **5**, 560–565.
- 14 R. Manasseh, K. Petkovic-Duran, P. Tho, Y. Zhu and A. Ooi, in *BioMEMS and Nanotechnology II*, SPIE, Brisbane, Australia, 2006, pp. 485–495.
- 15 C.-C. Hong, J.-W. Choi and C. H. Ahn, *Lab Chip*, 2004, **4**, 109–113.
- 16 H. Chen and J.-C. Meiners, *Appl. Phys. Lett.*, 2004, **84**, 2193–2195.
- 17 P. Vulto, T. Huesgen, B. Albrecht and G. A. Urban, *J. Micromech. Microeng.*, 2009, **19**(5), 077001.
- 18 P. Vulto, S. Podszun, P. Meyer, C. Hermann, A. Manz and G. A. Urban, *Lab Chip*, 2011, **11**, 1596–1602.
- 19 S. Chibbaro, E. Costa, D. I. Dimitrov, F. Diotallevi, A. Milchev, D. Palmieri, G. Pontrelli and S. Succi, *Langmuir*, 2009, **25**, 12653–12660.
- 20 S. Podszun, P. Vulto, H. Heinz, S. Hakenberg, C. Hermann, T. Hankemeier and G. A. Urban, *Lab Chip*, 2012, **12**, 451–457.
- 21 P. Vulto, G. Dame, U. Maier, S. Makohliso, S. Podszun, P. Zahn and G. A. Urban, *Lab Chip*, 2010, **10**, 610–616.
- 22 V. Mengeaud, J. Josserand and H. H. Girault, *Anal. Chem.*, 2002, **74**, 4279–4286.