DOI: 10.1002/cphc.200700566

An Acoustically Driven Microliter Flow Chamber on a Chip (μFCC) for Cell–Cell and Cell–Surface Interaction Studies

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A novel method for pumping very small volumes of liquid by using surface acoustic waves is employed to create a microfluidic flow chamber on a chip. It holds a volume of only a few μl and its planar design provides complete architectural freedom. This allows for the reconstruction of even complex flow scenarios (e.g. curvatures, bifurcations and stenosis). Addition of polymer walls to the planar fluidic track enables cell culturing on the chip surface and the investigation of cell–cell adhesion dynamics under flow. We demonstrate the flexibility of the system for application in many areas of microfluidic investigations including blood clotting phenomena under various flow conditions and the investigation of different stages of cell adhesion.

1. Introduction

Different varieties of parallel-plate flow chambers are important tools for the investigation of cell and protein behaviour under flow conditions. They help us gain better understanding in many areas of biology, biochemistry and biophysics. However, there remains a need to develop more versatile and smaller systems for the investigation of expensive or rare substances available only in small quantities or to mimic various microfluidic situations as are often encountered in nature. The miniaturization to the micro- and nanofluidic range has proven difficult, due to phenomena which become increasingly important at decreasing fluid volumes,[1] making mixing or pumping challenging. At low Reynolds numbers (Re) friction forces dominate over inertial forces and liquids appear to become more viscous, resulting in laminar flow and large flow resistances. Many physiological phenomena occur at these small length scales and are hence governed by microfluidic effects. One example occurs in the human vascular system where typical Reynolds numbers are significantly below Re = 1200—just beyond the transition between turbulent and laminar flow. The Reynolds numbers in terminal arteries with a radius of ~500 μm are only in the order of Re = 100[2]. Thus blood becomes increasingly difficult to pump or mix.

Over the last decade, there have been many interesting and innovative solutions to this problem, which have led to a variety of specialized microfluidic devices for cellomics and proteomics collectively known as micro total analysis systems (μTASs). These are reviewed elsewhere.[3] The fluids in these systems are often driven by external means (pneumatic pressure or syringe pumps) which, due to the introduction of an external aspect, can be difficult to handle and could provide a source of contamination.[4] Internal pumping mechanisms include electro–osmotic flow (EOF), valve-type micropumps using piezoelectric actuation, electrostatic pumps, diffuser pumps and the use of centrifugal force. Although these various solutions have catapulted us into a new dimension of miniaturized systems, they each have their own difficulties and drawbacks which have been thoroughly reviewed elsewhere.[4,5]

Driven by the needs described above, we have recently developed a novel internal pumping technique[6] for the simple and efficient transport, actuation and mixing of very small fluid volumes. The technique has already been successfully employed in many areas of research conducted in our laboratories[7–9] and in commercially available laboratory equipment (www.advalytix.com). The technique is based on the interaction of a fluid at the surface of a planar substrate and surface acoustic waves (SAWs) which produce effective acoustic streaming even in the sub-microliter range.[6]

We combined this acoustically driven nanopump with existing methods of hydrophobic/hydrophilic surface lithography to create two different types of microliter flow chambers on a chip (μFCC). They are suitable for a wide variety of applications, typically contain a sample volume of only 10 μl and are produced at low cost with conventional methods of planar technology. The first design relies on a completely planar (2D) geometry with a functionalized surface suitable for the investigation of the physics of soft objects (cells, proteins, polymers etc.) under hydrodynamic flow in narrow channels. Employing

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a relatively simple and flexible lithographic method with subsequent surface functionalization, we create chemically defined open channels which are able to confine small fluid volumes laterally. These virtual channels consist of laterally patterned hydrophilic lanes within a hydrophobic environment. Surface functionalization is a well-established process which requires no etching or moulding and leads to a particularly stable result.

The second acoustically driven μFCC we describe is a 3D version of the above flow chamber in which additional elastomeric structures are incorporated into the planar design. This allows for cell culturing on the chip surface and the subsequent investigation of protein–cell and cell–cell interactions under flow.

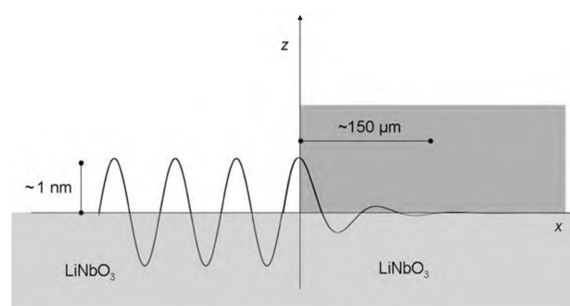

Figure 1. A surface acoustic wave (SAW), excited electrically on a 128°-Y $LiNbO_3$ substrate, propagates along the solid/air interface undamped until it interacts with a liquid (grey). The damped wave causes an acoustic streaming inside the liquid. Considering the total length of the channel (Figure 2), the surface wave acts as a localized pump, as its mechanical energy is absorbed by the liquid over only about 150 μm.

Experimental Section

The SAW Principle and Chip Design

In our flow chamber chips, we use SAWs to manipulate small amounts of liquid.[6] On a strong and anisotropic piezoelectric material, if we use a Y-Cut of $LiNbO_3$ with the crystal axes rotated around the X-axis by128° (128° Y-Cut), a SAW is initiated. The resulting Rayleigh wave has both longitudinal and transversal components and is excited to propagate along the X-axis of the crystal in order to avoid shear modes. The interdigitated transducers (IDT) used to deform the crystal are therefore oriented perpendicularly to the X-axis. The IDTs consist of a specially formed pair of gold electrodes which are deposited on top of the substrate by using standard lithography. The distance between the individual finger pairs of the electrodes is 15 μm which determines the excitation frequency of the SAW (see below). Applying a high-frequency voltage using a frequency generator (Rhode & Schwarz, SML 01, Munich, Germany) to the IDTs on the piezoelectric material results in the generation of a monochromatic and intense SAW which propagates along the surface at the speed of sound. The layout and geometry of the IDT determines the wavelength of the SAW which is typically in the micrometer range. The corresponding SAW frequencies [Eq. (1)] generally range between hundreds of MHz and GHz

$$f = c_{SAW}/\lambda \tag{1}$$

where c_{SAW} denotes the sound velocity of the substrate (~4000 $m\,s^{-1}$), and the wavelength λ is identical to the lithographically defined periodicity of the IDT (15 μm). Typical SAW amplitudes are less then one nanometer in the direction normal to the surface. To ensure biocompatibility and to protect the metallic IDT, the entire chip surface including the IDT is covered by 50 nm of SiO_2 using standard sputter techniques. The thin glass layer at the surface also ensures compatibility with existing chemistries for surface functionalization. Since the SAW is basically a sound wave travelling along a solid/air interface with poor impedance coupling (high difference in sound velocity) only very little energy is dissipated in this mode. However, when a SAW encounters a liquid placed on the upper half-space of the chip (Figure 1, right), energy is transmitted into the liquid due to the better matching in sound velocity between the solid and the liquid. This leads to so-called acoustic streaming within the fluid.[10] Due to the viscous attenuation, the amplitude of the SAW decays exponentially in the liquid within a characteristic length scale of a few micrometers. Therefore, considering the total length of a typical channel (40 mm) in our acoustically driven μFCC, the pump basically acts as a point-like source driving the liquid to the flow according to conservation of mass.

2D Chip Design: In order to functionalize the chip in 2D an octadecyltrichlorsilane (OTS) layer was adsorbed to the surface by incubating the chip in a silane–n-hexane mixture (24 μL of silane per 30 mL of hexane; Sigma–Aldrich, Taufkirchen, Germany) for 30 min. The silanization leads to complete hydrophobicity of the surface, with a wetting angle of 80° degrees. After protecting the entire silane layer by using a photo resist, the surface is masked with the desired hydrophilic lane structure and exposed to UV light. The developed chip is then etched using oxygen plasma and subsequently rinsed in an acetone bath to remove the left-over photo resist. This creates hydrophilic channels in which the fluid can flow without leakage, as if confined to a virtual tube, at even the highest flow velocities.

3D Chip Design: In order to design 3D fluidic channels we used the elastomer polydimethylsiloxane (PDMS) introduced before,[11–14] A 10:1 ratio of elastomer monomer:curing agent (Sylgard 184 Silicone Elastomer, Dow Corning, Germany) was mixed and allowed to cure for 1 h. The mixed PDMS was then poured directly onto a structured metal mold or an SU-8 photo resist structure (polymerisation took approximately 24 h) creating a flexible, yet stabile, 3D channel architecture.

Cells and Cell Culturing

For our experiments with the planar flow chamber chip, the lane was filled with phosphate-buffered saline (PBS). The experiments involving living cells were carried out with the human melanoma cell line M2 subclone A7, thoroughly described elsewhere (see ref. [15] and therein). Cells were cultured in modified Eagle's medium supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria), 1% streptomycin/penicillin and 0.35 $mg\,mL^{-1}$ of geneticin (Biochrom, Berlin, Germany). The cells were cultured in the medium for 2 days (until a confluent layer was achieved) at $T=37\,^{\circ}C$ and 5% CO_2 atmosphere. Cells were then removed from the cultured surface using a standard trypsinisation procedure, washed and resuspended in PBS before addition to the flow channel. Cells cultured directly on the chip were first seeded into the PDMS channels before the entire chip was flooded with media and allowed to grow to confluence. The chip surface was rinsed with PBS before experiments were performed. All experiments were performed at room temperature with a fluid vis-

cosity of approximately 1 cp. The cells were examined using a standard phase contrast microscope Axiovert 200 (Zeiss, Göttingen, Germany).

2. Results and Discussion

2.1. Planar Flow Chamber Chip (2D)

Although almost any geometry can be realized, we used chips with a channel of $w = 500$ µm wide and $L = 40$ mm long in the shape of a rectangle with rounded corners (racetrack) which holds 8–15 µl of fluid (Figure 2). The liquid within the lane is coupled to the SAW pump described above creating a homogenous laminar flow throughout the flow chamber. Only for a small portion (~150 µm) close to the SAW entrance we occasionally observed the formation of eddies.

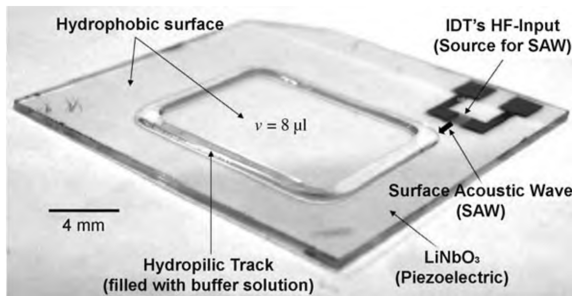

Figure 2. µ-Flow chamber chip. The vessel is modelled by a planar hydrophilic track on an otherwise hydrophobic piezoelectric substrate ($LiNbO_3$). A SAW (right) propagates from the IDTs towards the liquid-filled hydrophilic channel. At the entrance to the liquid it causes a localized pressure gradient which pumps the liquid. Due to the small scale of this microfluidic system (8 µl), the SAW creates a homogenous laminar flow along the channel.

We used this chip to examine the dynamic behaviour of the blood clotting protein known as the Von Willebrand factor (VWF) and its relationship to cell adhesion properties under flow. In our experiments, we were able to observe and describe a coil-stretched transition of the fluorescently labelled VWF which appears to be a crucial aspect in its physiological function. Our experiments examined the adhesion properties of this protein and their relationship to hydrodynamic forces in the microfluidic channel coated with collagen I. This system is meant to mimic the adhesion that is seen at sites of injury in small arteries upon exposure of subendothelial tissue (rich in collagen I). Our experiments thus provided insights into the mechanism of increased blood clotting that occurs at high-shear flow conditions in small arteries and is presented in detail elsewhere.[16–18]

Similar experiments mainly intended to demonstrate the suitability and validity of our system for the investigation of cell–protein interactions are presented in Figure 3. Here, melanoma cell adhesion to a collagen-coated chip surface was investigated. The results shown are comparable to those published in conventional flow chamber studies.[19] However, the volumes used in our setup are roughly 100 times smaller. As expected, the number of adhered cells decreases with increasing shear rate. Here, we would like to mention that the acting shear stress τ can be calculated from the applied shear rate which can be varied between 0 and 20000 s^{-1} and covers the whole physiological and pathological range. This is done by multiplying the shear rate $\dot{\gamma}$ with the viscosity η of the surrounding media [Eq. (2)]

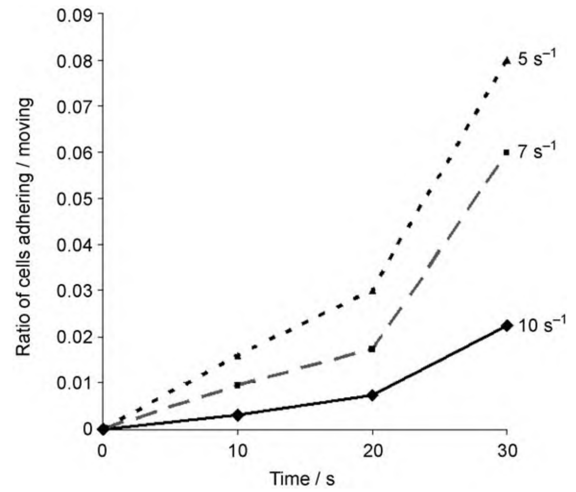

Figure 3. Cell–substrate interactions on a chip. Fraction of adhered vs freely floating cells as a function of time for three different shear rates are presented. The substrate is coated with collagen I.

$$\tau = \dot{\gamma}\eta \tag{2}$$

A shear rate of 10 s^{-1} therefore results in a shear stress of 10^{-2} Pa at room temperature in a PBS puffer.

2.2. Culture Flow Chamber Chip (3D)

Although our planar flow chamber chip is suitable to examine many relevant topics, it turned out not to perform optimally for cell cultures on its surface. In order to maintain a cell culture over several days, the entire chip surface has to be submerged in growth media, otherwise significant electrolyte and pH changes in the small fluid volumes prohibit cell growth. However, submergence of the planar chip in protein-rich media negatively influenced the channel architecture at the hydrophobic/hydrophilic interface due to protein adsorption onto the hydrophobic surface. This leads to unwanted inhomogeneous flow. To avoid this, we developed a 3D version of our system which easily allows for successful cell culturing while retaining the advantages of the planar system. We added "walls" to the channel by employing the fully transparent, biocompatible synthetic polymer polydimethyl-siloxane (PDMS, see Figure 4a)[20] onto the nonfunctionalized chip surface. We used a racetrack-shape 500 µm wide and 40 mm long channel- and a PDMS sheet with a depth of 1 mm (although thinner or thicker sheets can easily be achieved). The cell culturing process caused no destruction of the PDMS structure, nor was there leakage under the PDMS or growth of cells beyond the channel confines, evident by the sharp line marking the channel wall (Figure 4b). In addition, the cells appeared to divide

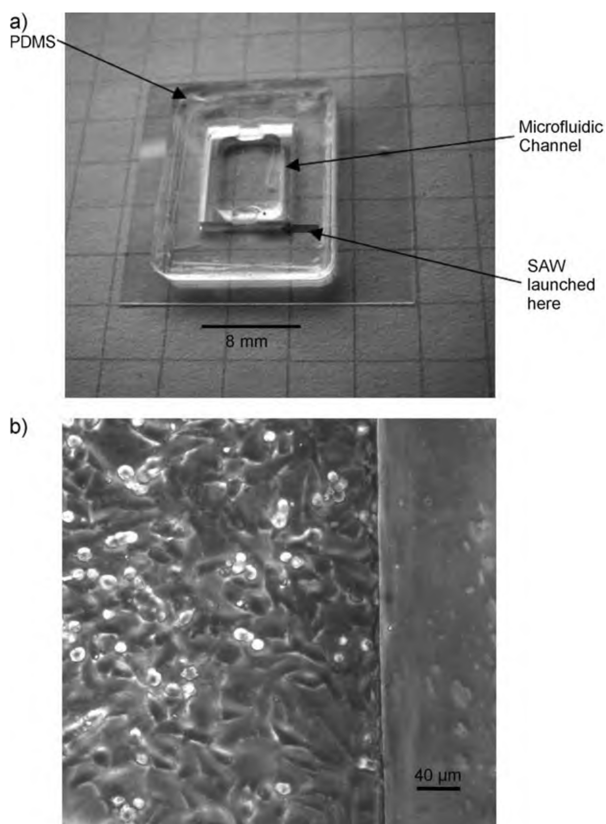

Figure 4. a) The 3D microfluidic channel made from PDMS in order to perform cell culturing directly inside the microfluidic channels. b) The cells form a confluent layer over the entire channel and do not grow across the boundaries (PDMS walls).

readily and were securely attached to the chip surface. The cultured cells also appeared largely unaffected by the SAW; they seemed healthy (as measured with tryphan blue vitality staining), were not changed in their gross morphology or reproduction time and remained securely attached to the channel floor.

To demonstrate the use of our system as an alternative to conventional flow chambers, we added individual cells (suspended in PBS) into the channel with the cultured cell monolayer. The picture series in Figure 5 shows these individual cells flowing over the confluent and intact cell monolayer (see Supporting Information Movie S1). The same cell is tracked at three different points in time (0, 1 and 2 seconds). Note that the underlying layer of cells is neither moving nor changing in its overall appearance, clearly indicating the monolayer integrity. The flow can be observed easily due to the chip's transparency, allowing the qualitative and quantitative separation of different adhesion states: flowing, rolling and firm adhesion. On the right-hand side of Figure 5 two cells at different velocities are seen passing each other. This demonstrates that the cells do not settle or accumulate in one plane, but are well-distributed over the entire depth of the channel. We do not intend to discuss the cell adhesion of melanoma cells on various substrates in detail; rather, we want to illustrate the suitability and validity of our microfluidic systems for the investigation of cell–protein and cell–cell adhesion phenomena under flow conditions.

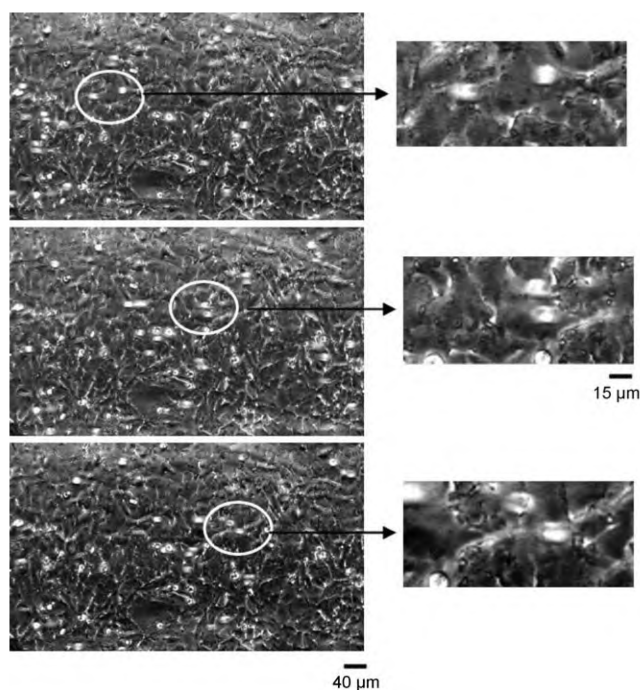

Figure 5. Cell–cell interactions on a chip. Two cells flowing over the intact and immobilized confluent cell layer are shown. The optical transparency of the piezoelectric substrate material and the continuous channel design enable the cells to be tracked for an unlimited amount of time. The upper cell moves at a different velocity than lower cell, indicating that the latter is farther away from the surface.

3. Conclusions and Outlook

We have developed a novel microfluidic flow chamber on a chip by using the phenomenon of surface acoustic streaming. Evidence is provided that these systems are suitable for tackling several questions encountered in modern life science. Both our 2D and 3D flow chamber chips have been demonstrated to be an efficient and versatile technique with many advantages over conventional flow chambers. One particular advantage of the system is its small volume (from a few µL upwards) allowing the investigation of costly and/or rare substances. Further advantages are the open and easily accessible track, complete optical transparency, flexible lane geometry and a large range of achievable flow velocities while maintaining a homogenous laminar flow with predictable shear rates. In addition, the channel can easily be coated with different surfaces of interest (collagen, poly-l-lysin, lipid membranes, adhesion proteins, endoprosthetic implant surfaces, etc.) without disrupting the track architecture.

The complexity of our vascular system and the prominence of vascular diseases such as arteriosclerosis, thrombosis and haemophilia emphasises the need for an in vitro vascular model that can adequately mimic both the biological and physical aspects of blood flow, particularly in the smallest arteries and veins. We have already succeeded in cultivating endothelial cells in the channels of our flow chamber chip as a first step towards realising an in vitro vascular model. We plan on using this system in the future to examine various blood

phenomena and the effects of different vessel geometries (e.g. stenosis, bifurcations, aneurysm).

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft DFG (SFB 486), the Fond of the Chemical Industry and the Elite Netzwerk Bayern (CompInt) as well as technical support from Advalytix AG, Brunntal (Germany) and T. Frommelt are acknowledged. This work was also supported by the Interdisziplinäre Medizinische Forschung IMF, Münster, Germany (SFP TP A13, grant S. W. S). Further financial support of the German Excellence Initiative via the Nanosystems Initiative Munich (NIM) is gratefully acknowledged.

Keywords: materials science · mechanical properties · microfluidic flow chamber · proteins · surface acoustic waves

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