Development of a microfluidic system based on polycarbonate as an artificial blood capillary vessel for medical application in cancer research[a]

Taleieh Rajabi[1,b,c], Volker Huck[2,c], Ralf Ahrens[1], Marie-Christin Apfel[2], Seoung-Eun Kim[1], Stefan W. Schneider[2], Andreas E. Guber[1]

[1]*Karlsruhe Institute of Technology (KIT), Institute of Microstructure Technology, (IMT), Hermann-von-Helmholtz-Platz 1, 76344 Eggenstein-Leopoldshafen, Germany*

[2]*Heidelberg University, Medical Faculty Mannheim, Experimental Dermatology, Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany*

We present for the first time a microfluidic system based on polycarbonate which acts as an artificial blood capillary vessel to examine the migration of cancer cells from the blood system into the surrounding tissue (extravasation). The microfluidic system consists of two micro structured layers which are separated by a porous membrane. The upper channel represents the blood vessel system, while the lower layer is filled with matrix components mimicking the surrounding tissue. Inside the blood vessel system the surface of the membrane is coated with a monolayer of human endothelial cells mimicking the vessel wall. The two micro channel systems are fabricated by hot embossing of polycarbonate which was chosen due to its transparency, high strength, toughness and very good biocompatibility and assembled using a thermal bonding process. For our experiments, the adherent endothelial monolayer in the upper channel was superfused with a cancer cells containing solution addressing the

[a] Contributed paper, published as part of the Proceedings of the 3rd European Conference on Microfluidics, µFlu'12, Heidelberg, December 03-05, 2012, Germany

[b] Electronic mail: taleieh.rajabi@kit.edu

[c] Taleieh Rajabi and Volker Huck contributed equally to this work

microscopy observation of the process of extravasation. The important feature is that all processes take place under continuous laminar flow conditions. We used computer-aided simulations of the flow behavior of cancer cells considering cell diameter, density and viscosity of the blood. We examined the entire migration steps of intravascular cancer cells from the overflowing, the unstable attachment, the firm adhesion and diapedesis up to migration into the matrix using live cell and fluorescence microscopy. Therefore we could show that a suitable selection of the membrane and the distribution of pore density, respectively, are of major importance for later endothelial cultivation and migration of cancer cells.

I. INTRODUCTION

The most hazardous characteristic of cancer is its ability to form metastatic tumor cells. If these cells penetrate the vessel system (intravasation), they can be transported to distant regions where they may settle and colonize other organs as metastases (cf. Figure 1). The aim of this work is to examine the entire migration steps of cancer cells penetrated into the blood stream from overflowing, unstable attachment, firm adhesion and diapedesis up to migration into the matrix (extravasation) using live cell and fluorescence microscopy.

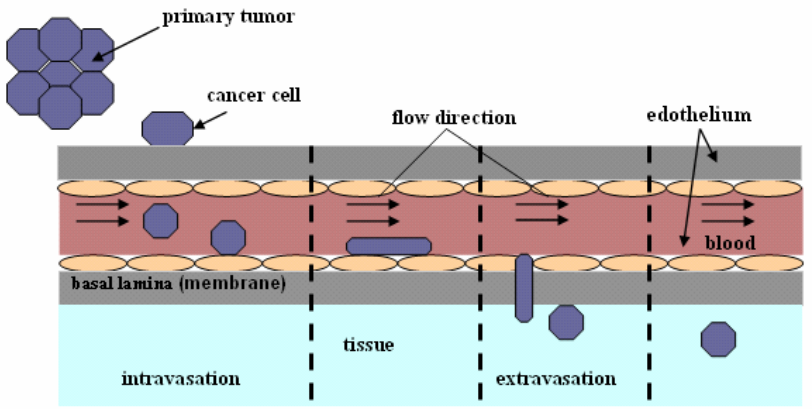


Figure 1: Schematic illustration of the steps of metastasis inside the blood capillary

Up to now other groups have performed experiments focussing on single steps of the process of cancer cell attachment, migration and extravasation. A commonly utilized technique in biomedical application is the Boyden chamber [1, 2] to investigate chemotaxis and migration under static conditions [3]. To address the attachment and interaction of cancer cells with the vessel wall other groups utilize a chamber system from ibidi® [4] for medical experiments under flow conditions [5, 6]. Furthermore channel systems made from PDMS have been used for the blood analysis [7]. However, this material is not suitable for the investigation of the cell growth and migration of cancer cells [8].

To investigate the entire process of cancer cell extravasation these techniques had to be combined utilizing an efficient fabrication technology with respect to biocompatibility, rheological properties of the vasculature and a biomedical transmigration readout, forming a novel microfluidic two-channel system. To mimic the conditions in the human microvasculature we use flow channels with heights in the range of 50 μm or 100 μm [9].

For the fabrication of the microfluidic system we use the polymer polycarbonate. Due to its properties such as biocompatibility, high strength and transparency, polycarbonate is a well known and most widely used polymer in the field of biomedical research. An additional aspect for the selection of polycarbonate was the required consistency of the seperating membrane, forming the basal lamina of the artificial blood vessel wall and the remaining microfluidic system. The complete system is fabricatetd by the combination of hot embossing [10] and thermal bonding [11, 12].

The work has been accompanied by numerical simulations of the flow behavior of the cancer cells inside the artificial blood vessel.

II. EXPERIMENTAL SETUP

Since the investigations of the migration steps of cancer cells should be performed under flow conditions, a microfluidic system is required which acts as an artificial blood

vessel. To mimic the physiological conditions of the capillary part of the human vasculature it is necessary to pay particular attention to the microfluidic dimensions of this artificial blood vessel: The physical conditions of fluids at this microscale level are characterized by a low Reynolds number indicating the ratio of inertial versus viscous effects. At the relevant scale for cancer cell – endothelium interaction, i.e. a vessel diameter range of 5 to 200 µm, inertial effects become irrelevant and are overwhelmed by friction effects [13]. These occasionally counterintuitive conditions as well as the determination of the relevant flow velocities inducing typical microvascular wall shear rates [14] have to be taken into consideration in the development of a suitable system for the investigation of cancer cell extravasation. Furthermore, the developed system should be optically transparent to allow microscopically surveillance and should be composed of biocompatible materials, fabricated using standard replication techniques such as injection molding or hot embossing.

A. Concept

The microfluidic system is composed of two layers, an upper channel system which consists of two sets of three micro structured channels with a width of 300 µm each, simulating the blood vasculature, and the lower layer, which consists of two large channels that are filled with artificial tissue (patent pending). Both layers are separated by a porous membrane with a specific pore density distribution (cf. Figure 2). The suitable selection of the membrane and the distribution of pore density, are of major importance for the later endothelial cultivation and migration of cancer cells. To investigate the influence of the vessel diameter, we utilize two systems with a height of 50 µm and 100 µm, respectively.

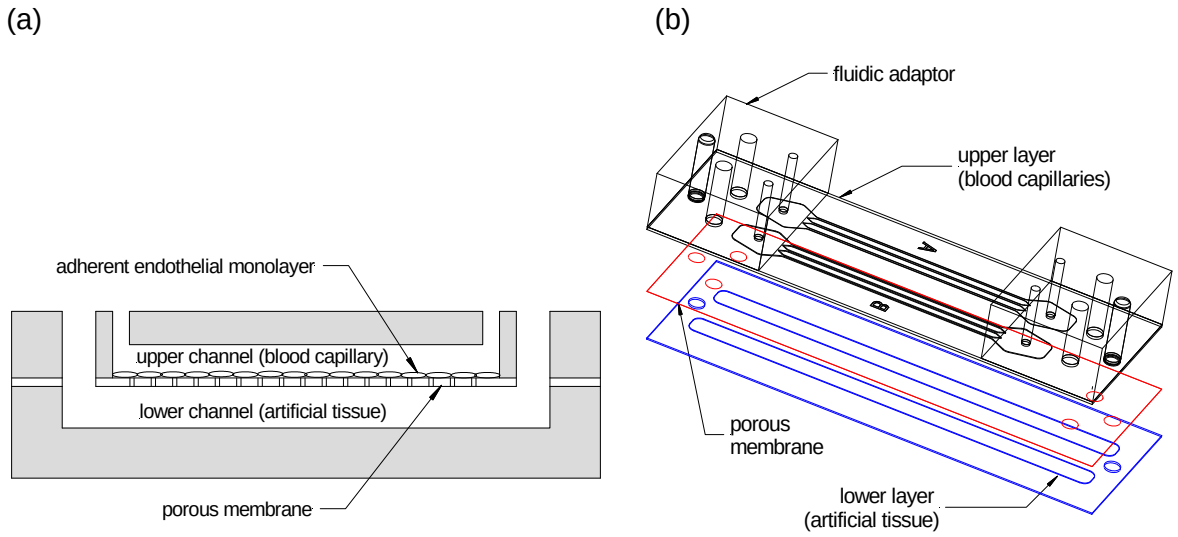


Figure 2: Schematic view of the artificial blood vessel system: (a) cross section (b) exploded view

B. Material selection

In general, the polymer material used for the construction of the fluidic chip system has to be optically transparent, free of interfering autofluorescence and suitable for cell attachment. To facilitate the assembly of the system by thermal bonding, the membrane and the other components of the system should be made of the same polymer.

Looking for an appropriate polymer material, different polymer samples have been tested on auto fluorescence, cell adhesion and biocompatibility. To investigate the cell adhesion, small polymer slides were autoclaved at 110 °C for 15 min and then two specialized cell culture media (M199: Earle's salts, L-glutamine, sodium bicarbonate, 10% FCS, 1% heparin, 1% Penicellin/Streptomycin, 1% bovine eye growth factor and EGM-2: Endothelial basal medium 2 (Lonza) supplemented with 2% FCS, 0.4% hFGF, 0.1% VEGF, 0.1% R3-IGF-1, 0.1 % hEGF, 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100) were applied which were supplemented with stimuli for adequate growth conditions for the cultivation of human endothelial cells. As shown in Figure 3, adhesion of human endothelial cells on COC (Cyclic Olefin Copolymer) was the best using EGM-2 cell culture

medium, while on PS (Polystyrene) and PC (Polycarbonate) cell growth and adhesion properties were reduced. Using the M199 cell culture medium, the cell adhesion on PC was the best, followed by COC, but there was no cell adhesion on PS (Data not shown). Since porous PC foils are commercially available, polycarbonate was chosen for this work.

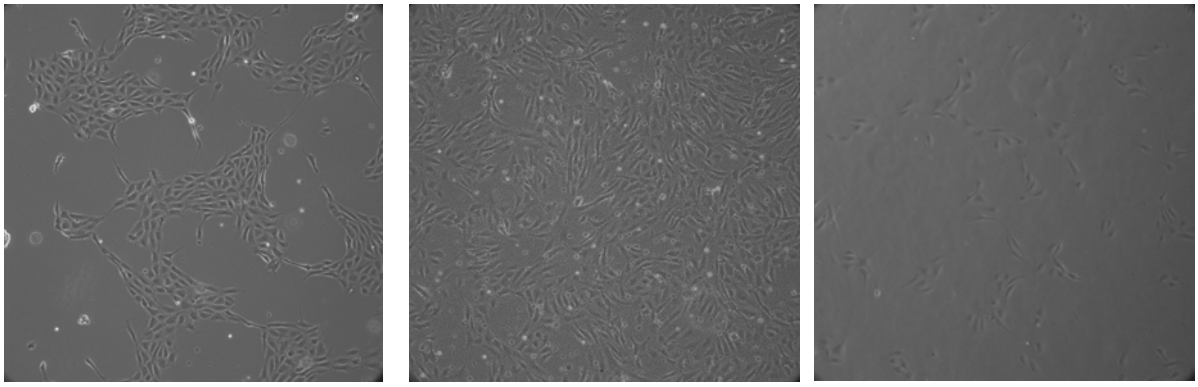


Figure 3: Micrographs of the cell adhesion using EGM-2 medium on different polymer surfaces

C. Fabrication of the membrane

As mentioned in chapter 2.1 the membrane needs a certain pore density and well-defined pore diameters. In the market you will find diverse porous polycarbonate membranes which are commonly fabricated using the nuclear track method. Here a sheet of polycarbonate is irradiated with argon or xenon ions, causing tracks of damaged polymer molecules inside the foil. These damaged polymer structured can be removed in a later wet etching process using for example a NaOH-solution at 50°C [15]. While the radiation intensity affects the pore density distribution, the pore diameter is controlled by the etching parameters.

In addition to membranes with a pore density of $10^5/cm^2$ and a pore diameter of 5 µm which are commercially available, we have also tested membranes with a reduced pore density of $10^3/cm^2$ or membranes with spatially variable pore density. Moreover we have

used membranes with a regular pore distribution fabricated by laser ablation or a dry etching process.

Prior to the experiments with the microfluidic two-channel system, the porous membranes have been examined in regard to the cell adhesion using a special test set up as shown in Figure 4.

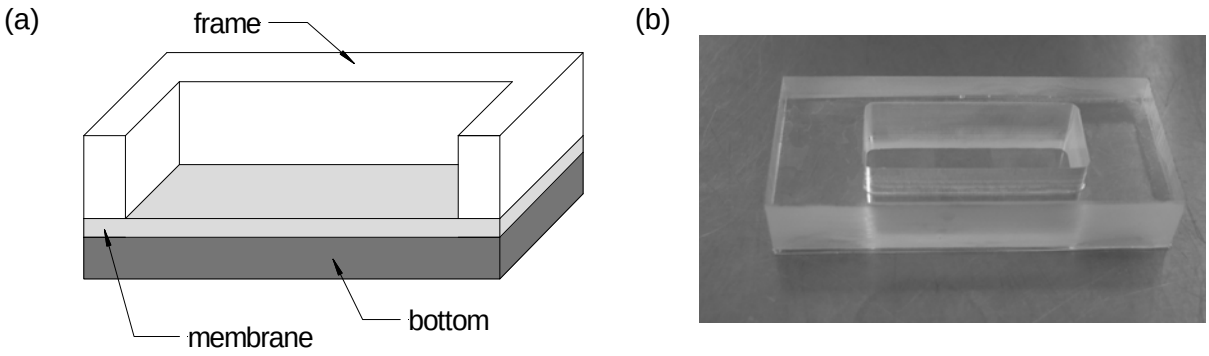


Figure 4: (a) Schematic of the membrane test system (b) photograph of the membrane test system

D. Fabrication and assembly

The two layers of our microfluidic system have been produced by hot embossing [9] of polycarbonate foils (Makrofol DE 1-1, Bayer Material Science). The embossing tool was fabricated by precision micro-milling of brass using diamond tools to achieve smooth edges.

For the hot embossing, an appropriate sheet of polycarbonate is positioned on top of the embossing tool (cf. Figure 5 (a)) inside a vacuum chamber. Then the counter plate is lowered until it touches the polymer sheet to improve the thermal contact and the embossing tool and the counter plate are heated up to the forming temperature which is above glass transition temperature of the polymer. Now the embossing force is increased to press the softened polymer into the embossing tool (cf. Figure 5 (b)). In the next step the complete system is cooled down to the demolding temperature without changing the embossing force. Finally the system is opened by lifting the counter plate, whereby the component is demolded as shown in Figure 5 (c).

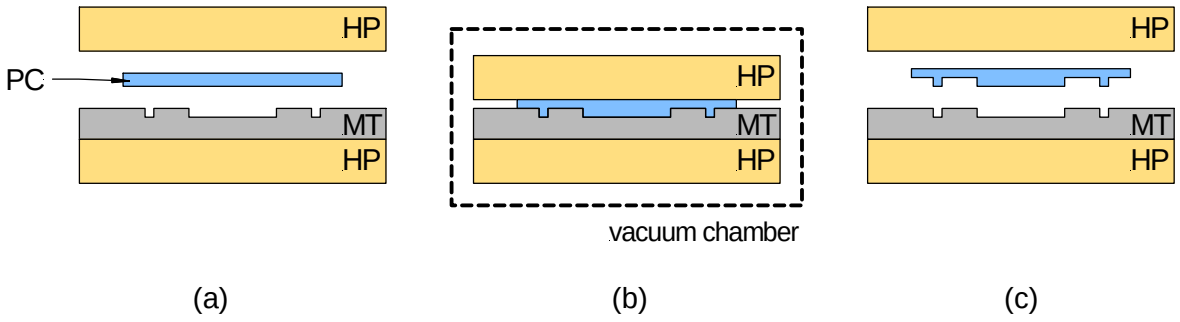


Figure 5: Schematic of the hot embossing process (HP: Hot plates, MT: Molding tool, PC: PC sheet). (a) Loading a PC sheet into the machine and preheating (b) Applying the embossing force to press the softened PC into the molding tool under vacuum (c) Cooling down to deforming temperature and deforming of the hot embossed PC component.

Afterwards the corresponding molded parts and the membrane are assembled to the final microfluidic system using a two-step thermal bonding process. By this procedure, first the layer with the blood vessel system and the membrane are heated up to glass transition temperature while being pressed together for several minutes. In the second step this new subsystem is bonded to the artificial tissue layer in the same manner (cf. Figure 6). To ensure a good optical transparency of the surface, the components are pressed together using chromes steel plates. The additional silicone rubber layer is used to compensate possible variations of the thickness of the microstructured PC layers. Finally, the microfluidic system is connected with fluidic adaptors using an UV-activated adhesive to improve the access to the individual channel systems.

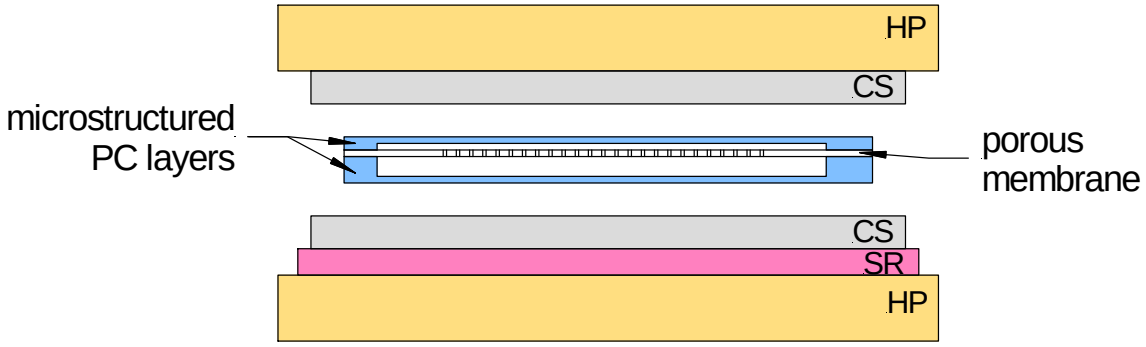


Figure 6: Schematic of the thermal bonding process.
(HP: hot plates, CS: chromed steel plates, SR: silicone rubber)

III. SIMULATION OF THE FLOW BEHAVIOR OF THE TUMOR CELLS

Prior to the fabrication of the molding tools the design of the artificial blood vessel was optimized using numerical simulations of the flow behaviour of the tumor cells inside the channel system. The most important question was the calculation of the cell distribution to be expected.

The numerical simulations of the flow of the cancer cells have been performed using ANSYS® Fluent. They are based on parameters such as weight, diameter and flow rate of the cancer cells and density and viscosity of the blood, respectively. The geometrical height of the channel was reduced to 80 % due to the fact that during the biological experiments the channel bottom is coated with a monolayer of human endothelial cells seeded on gelantine.

To reduce the processing time the model of the channel system was simplyfied without loosing its original properties. In addition the simulation of the entire system was divided into three zones, the input and the output area and the channel area as shown in the upper section of Figure 7.

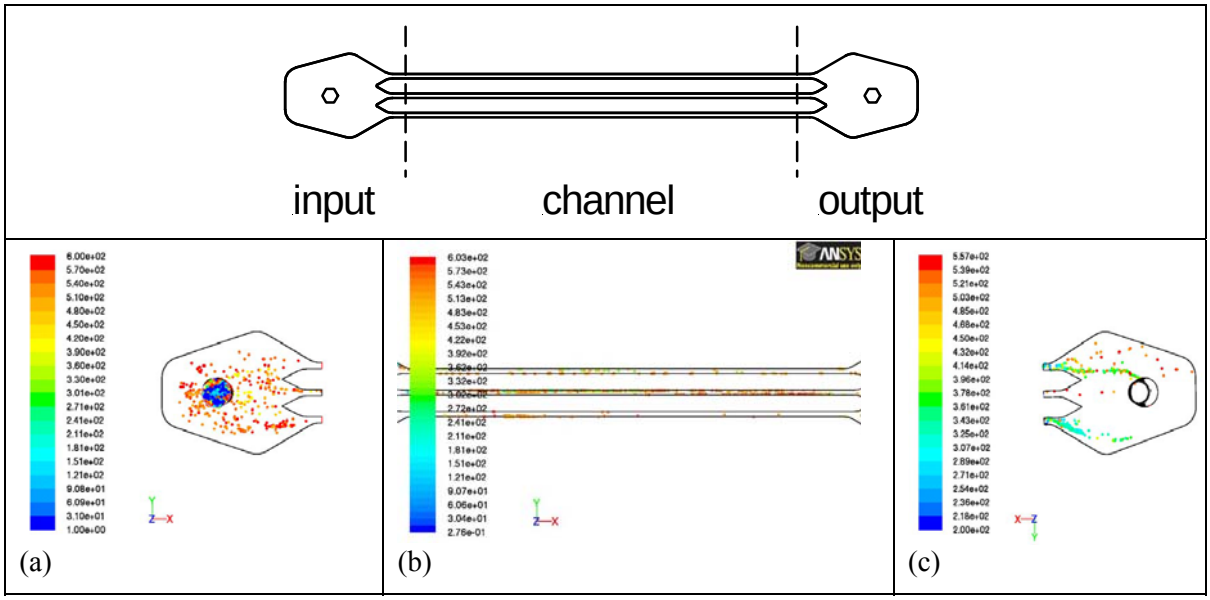


Figure 7: Results of the numerical simulations for the different zones; the color scale represents the different times

The numerical simulations were started under the assumption that 168,000 cancer cells are injected into the input area of the channel system. Then the cell distribution after 600 s within the input zone was calculated. Due to the diffusion the cells are distributed uniformly over the input area as shown in Figure 7 (a).

Those cells, which can leave the input zone, will enter the channel system. However, the cell distribution over the 3 individual channels is non-uniform with an accumulation inside the central channel (cf. Figure 7 (b)). Due to the velocity profile of the blood flow, the cells next to the channel walls are very slow. In reality they stick to the endothelial monolayer and may even penetrate this layer. All the other cells will leave the channel system because of the existing blood flow.

Most of the cells which enter the output area will leave this zone through the outlet tube (cf. Figure 7 (c)). However, due to the resulting pressure difference, a small amount of cells may be redirected back into the channel system, where they increase the number of cells

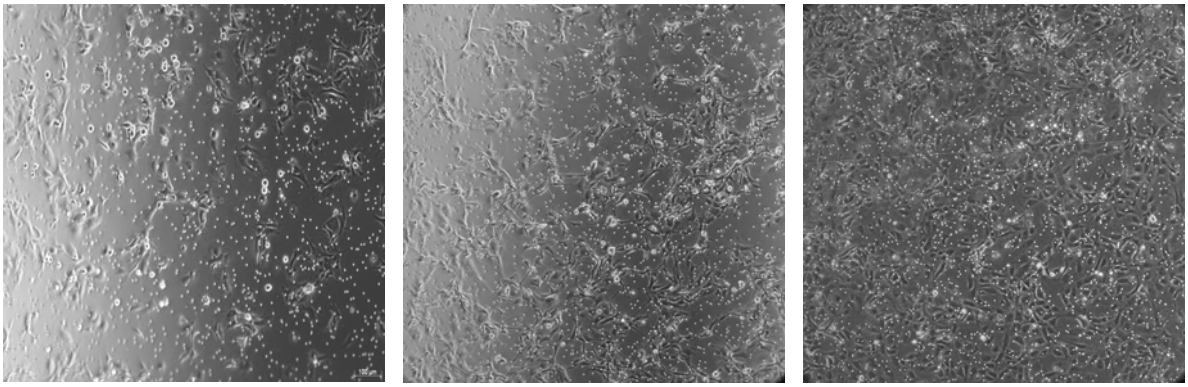
which are attached to the endothelial monolayer. All the other cells will again be affected by the blood flow and be carried back to the output area.

Summarized, the numerical simulations have shown that about 9% of the cancer cells injected into the microfluidic system will remain in the channel system and might adhere to the endothelial monolayer. However, only a small amount of these cells will finally penetrate this layer.

IV. BIOLOGICAL EXPERIMENTS WITH MICROFLUDIC CHIP AND MEMBRANES

A. Biocompatibility of the porous membranes

For biomedical application of the microfluidic chip system in the field of cancer cell transmigration, it was necessary to utilize a porous membrane for seeding endothelial cells. While PC was identified to be the optimum regarding both biocompatibility and adequate production technology as mentioned in chapter 2.2, we tested endothelial cell growth and adhesion on PC membranes with an aimed pore density of 10^5 per cm^2. All chips were precoated with 300 µl of 0.5% gelatine for 30 min in an incubator at 37 °C and 0.5% CO_2. A solution of 150,000 cells in 300 µl of pre-equilibrated special cell culture medium EGM-2 was seeded onto the gelatine-coated membrane. Finally, the membranes were observed using a phase contrast microscope to validate the cell growth properties also on porous membranes. Representative micropraphs of a porous PC membrane are shown in Figure 8 confirming an adequate biocompatibility of porous PC membranes.



(a) (b) (c)

Figure 8: Micrographs of the cell growth and the cell adhesion on porous PC membranes with a pore density of $10^5/cm^2$ at 1 h (a), 6 h (b) and 24 h (c) after the cell seeding (Edge length of the pictures: 1.4 mm).

B. Biomedical experiments with the microfluidic chip

For the biomedical experiments, the lower channels of the system A and B were filled with matrix components as described in the cell culture protocol (here: 0.3 ml of 20% gelatine, heated to 50 °C in a 1 ml syringe). Then the microfluidic dual-chamber system was incubated for 1 h at 37 °C and 5% CO_2 under sterile conditions. Thereafter, the upper channels were gently rinsed with distilled ultra pure water, filled with pre-incubated cell culture medium EGM-2 and placed in the incubator at 37 °C and 5% CO_2 for 24 h. After this pre-treatment, human endothelial cells were seeded into the upper channel (480,000 cells per channel in 100 µl of EGM-2 medium and grown to confluence. After 48 h, a homogeneous endothelial monolayer grew onto the upper side of the porous membrane. For validation, living endothelial cells were stained with cell trace calein red-orange (Invitrogen, 50 µg dissolved in 50 µl DMSO) as shown in Figure 9 (a).

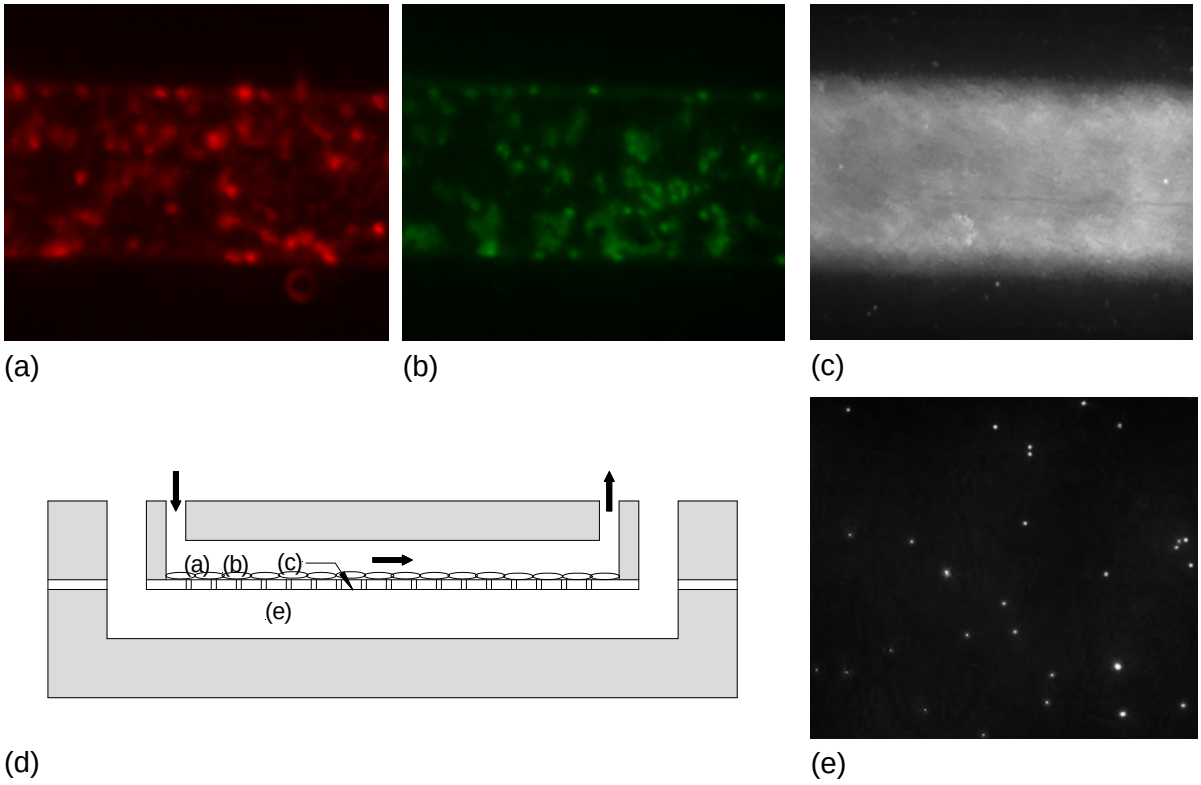


Figure 9: Fluorescence micrographs of stained cells inside the artificial blood vessel system; (a) living endothelial cells stained with cell trace calein red-orange on top of the porous membrane inside the upper channel; (b) fluorescence signal from rolling or adherent cancer cells at membrane level inside the upper channel; (c) fluorescence signals of transmigrated cancer cells marginal below the porous membrane (white dots) - note that this signal is overlapped by out of focus-fluorescence from floating cancer cells inside the upper channel (white noise); (d) schematic of the focal planes of the micrographs inside the microfluidic chip; (e) fluorescence signal of transmigrated cancer cells inside the lower channel between the matrix components. (All pictures have the same scale; channel width: 300 µm)

For the first investigation of tumor-endothelial interaction and tumor extravasation, the microfluidic system was mounted on an inverted microscope (AxioObserver Z.1, Zeiss GmbH, Jena, Germany) equipped with an incubation chamber and connected to a syringe pump. Subsequently, the upper channel was rinsed with a cancer cell containing isoosmolar solution of approximately 10^7 tumor cells per ml stained with Cell Trace calcein green (Invitrogen, 50 µg dissolved in 50 µl DMSO) at distinct shear rates, simulating the fluidic

conditions of human microvasculature (representing a shear rate of 1 dynes/cm^2). Representative fluorescence images of the floating cancer cells were taken after 5 minutes of perfusion with a fixed exposure time using a CCD camera AxioCam MRm operated by AxioVision software V4.8.2 (both Zeiss GmbH, Jena, Germany). Distinct fluorescence signals from rolling or adherent cancer cells in direct contact with the endothelial layer were conspicuously visible at the membrane level (Figure 9 (b), specific excitation of cell trace green). Next to the bottom side of the porous membrane fluorescence signals of transmigrated cancer cells have been observed (Figure 9 (c)) as well as a smear of fluorescence signals due the floating cancer cells inside the upper channel. In concordance with our simulations, only a small part of the cancer cell fraction came in contact with the endothelial layer. To verify the migration of the cancer cells through the endothelial monolayer on the gelatine-coated porous membrane into the lower matrix channel and therefore the process of extravasation, we took fluorescence images of the lower channel after 10 minutes of perfusion and found transmigrated cancer cells between the matrix components shown in Figure 9 (e).

V. CONCLUSIONS

The development of this microfluidic channel system was mainly focused on the rheological simulation of the microvascular system. The combination of production technology, channel structure, tuneable flow parameters and functionalized membrane enables unique new medical and scientific applications. This microfluidic system acts as an artificial blood vessel providing a direct insight into the process of cancer cell extravasation under flow conditions.

For future experiments it is planned to integrate gold electrodes on top of the membrane which can be used as impedance sensors to detect the endothelial growth. The impedance spectroscopy, especially the Electric Cell-substrate Impedance Sensing ECIS® is an accurate method to examine the electrical properties of membranes or cell layers [16].

Besides the investigation of cell attachment or cell spreading ECIS also allows the examination of migration processes [17]. This implementation might improve the reliability and the quantitative analysis of our microfluidic experimental setup.

ACKNOWLEDGEMENTS

This work was partly carried out with the support of the Karlsruhe Nano Micro Facility (KNMF, www.kit.edu/knmf), a Helmholtz Research Infrastructure at Karlsruhe Institute of Technology (KIT, www.kit.edu).

REFERENCES

[1] Boyden, S. (1962). The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J Exp Med*, **115**, 453-466.

[2] Toetsch, S., Olwell, P., Prina-Mello A. & Volkov, Y. (2009). The evolution of chemotaxis assays from static models to physiologically relevant platforms. *Integr. Biol.,* **1,** 170–181.

[3] Fu, B. H., Wu, Z. Z. & Qin J. (2011). Effects of integrin α6β1 on migration of hepatocellular carcinoma cells. *Mol Biol Rep*, **5**, 3271-6.

[4] Hoffmann, B., Merkel, R. & Rädler, U. (2007). Zellmikroskopie unter in vivo-nahen Bedingungen. *BIOspektrum*, **13**, 401-403.

[5] Strell, C., Lang, K., Niggemann, B., Zaenker, K. S. & Entschladen, F. (2011). Surface molecules regulating rolling and adhesion to endothelium of neutrophil granulocytes and MDA-MB-468 breast carcinoma cells and their interaction. Cell Mol Life Sci, **24**, 3306-16.

[6] Song, J. W., Cavnar, S. P., Walker, A. C., Luker, K. E., Gupta, M., Tung, Y.-C., Luker G. D. & Takayama, S. (2009). Microfluidic Endothelium for Studying the Intra-vascular Adhesion of Metastatic Breast Cancer Cells. *PLoS One*, **4**, e5756.

[7] Zengel, P., Nguyen-Hoang, A., Schildhammer, Ch., Zantl, R., Kahl, V. & Horn, E. (2011). μ-Slide Chemotaxis: A new chamber for long-term chemotaxis studies. *BMC Cell Biology*, 12:21, doi:10.1186/1471-2121-12-21, http://www.biomedcentral.com/1471-2121/12/21.

[8] Webb, K., Hlady, V. & Tresco, P. A. (2000). Relationships among cell attachment, spreading, cytoskeletal organization, and migration rate for anchorage-dependent cells on model surfaces. *J. Biomed. Mat. Res.*, **49**, 362-368.

[9] Pries, A.R., Secomb T.W. & Gaehtgens P. (1996). Biophysical aspects of blood flow in the microvasculature. *Cardiovasc Res*., **32**, 654-667.

[10] Worgull, M. (2009). Hot embossing: Theory and technology of microreplication. William Andrew, Oxford.

[11] Tsao, C.-W. & DeVoe, D. L. (2009). Bonding of thermoplastic polymer microfluidics. *Microfluid Nanofluid*, **6**, 1–16, DOI 10.1007/s10404-008-0361-x.

[12] Petrova-Belova, L. (2010). *Mehrlagige mikrofluidische Systeme aus Polymeren zur zweidimensionalen Kapillarelektrophorese*, Karlsruhe Institute of Technology (KIT), KIT Scientific Publishing, ISBN 978-3-86644-518-5.

[13] Squires, T. M. & Quake, S. R. (2005). Microfluidics: Fluid physics at the nanoliter scale. *Rev. Mod. Phys.*, **77**, 977–1026.

[14] Kroll, M.H., Hellums, J.D., McIntire, L.V., Schafer, A.I. & Moake J.L. (1996). Platelets and shear stress. *Blood*, **88**, 1525-1541.

[15] Chtanko, N., Toimil Molares, M. E., Cornelius, T., Dobrev, D. & Neumann, R. (2002) *Ion track etching in polycarbonate for nanowire synthesis*. Gesellschaft für Schwerionenforschung (GSI), http://www.alt.gsi.de/informationen/wti/library/scientificreport2002/files/132.pdf, 11.07.2010.

[16] Keese, C. R., Bhawe, K., Wegener, J. & Giaever, I. *Real-Time Impedance Assay to Follow the Invasive Activities of Metastastic Cells in Culture*, BioTechniques **33**:840-850 (October 2002)

[17] Ren, J., Xiao, Y., Singh, L.S., Zhao, X., Zhao, Z., Feng, L, Rose, T.M., Prestwich, G.D. & Xu, Y. *Lysophosphatidic Acis Is Constitutively Produced by Human Peritoneal Mesothelial Cells and Enhances Adhesion, Migration, and Invasion of Ovarian Cancer Cells*, Cancer Res 2006; **66**: (6) (2006)

Figure Captions:

Figure 1: Schematic illustration of the steps of metastasis inside the blood capillary

Figure 2: Schematic view of the artificial blood vessel system: (a) cross section (b) exploded view

Figure 3: Micrographs of the cell adhesion using EGM-2 medium on different polymer surfaces

Figure 4: (a) Schematic of the membrane test system (b) photograph of the membrane test system

Figure 5: Schematic of the hot embossing process (HP: Hot plates, MT: Molding tool, PC: PC sheet). (a) Loading a PC sheet into the machine and preheating (b) Applying the embossing force to press the softened PC into the molding tool under vacuum (c) Cooling down to deforming temperature and deforming of the hot embossed PC component.

Figure 6: Schematic of the thermal bonding process. (HP: hot plates, CS: chromed steel plates, SR: silicone rubber)

Figure 7: Results of the numerical simulations for the different zones; the color scale represents the different times

Figure 8: Micrographs of the cell growth and the cell adhesion on porous PC membranes with a pore density of $10^5/cm^2$ at 1 h (a), 6 h (b) and 24 h (c) after the cell seeding (Edge length of the pictures: 1.4 mm).

Figure 9: Fluorescence micrographs of stained cells inside the artificial blood vessel system; (a) living endothelial cells stained with cell trace calein red-orange on top of the porous membrane inside the upper channel; (b) fluorescence signal from rolling or adherent cancer cells at membrane level inside the upper channel; (c) fluorescence signals of transmigrated cancer cells marginal below the

porous membrane (white dots) - note that this signal is overlapped by out of focus-fluorescence from floating cancer cells inside the upper channel (white noise); (d) schematic of the focal planes of the micrographs inside the microfluidic chip; (e) fluorescence signal of transmigrated cancer cells inside the lower channel between the matrix components. (All pictures have the same scale; channel width: 300 μm)