Novel designs and technologies for cell engineering

Karina Ziółkowska, Michał Chudy, Artur Dybko, Zbigniew Brzózka

Department of Microbioanalytics, Institute of Biotechnology, Warsaw University of Technology,
e-mail: kziolkowska@ch.pw.edu.pl

Microfluidic devices, such as lab-on-a-chip systems, are highly advantageous for cell engineering and cell based assays. It is a particularly useful approach for development of the *in vitro* cellular systems mimicking the *in vivo* environment. In this paper, a novel lab-on-a-chip device for three-dimensional human cell culture and anticancer drug testing is presented. Cells were cultured as Multicellular Tumor Spheroids (MCTS) — the best cancer tumor model developed so far. Different designs were tested and novel technique of microfabrication in poly(dimethylsiloxane) was developed. MCTS were cultured in a system of polymeric microwells, with the network of microfluidic channels for culture medium flow. Design included optimal shear stress and proper nutrients supply for cultured cells. Final design provided MCTS culture for four weeks with the homeostasis-like state achievement, which is characteristic for the *in vivo* situation.

Keywords and phrases: lab-on-a-chip, multicellular tumor spheroid, cancer, *in vitro* cell culture, cell engineering.

Introduction

One of the major goals of modern cell engineering is development of cellular systems mimicking the *in vivo* environment. The *in vivo*-like systems are required for basic studies on mammalian and human physiology as well as for toxicology and drug development applications. Nowadays, experiments are performed using simple *in vitro* models (such as monolayer cell culture) which hardly correspond with the *in vivo* conditions [1–3]. Cells isolated from a body lack specific interactions, which results in significant differences in morphology, physiology and gene expression [4, 5]. Therefore novel techniques of cell culture and testing, more closely mimicking the *in vivo* environment, are required. Different approaches on the subject have been investigated: (1) texture, structure and chemical modifications of substrates for cell adhesion [6], (2) three-dimensional culture using protein or polymeric scaffolds [7], ECM analogues [3] and spontaneously formed cell aggregates [4, 8, 9], (3) vascular-like systems for nutrient and oxygen supply for maintenance of tissue explants or extended three dimensional cultures [10], (4) controlling of the hydrodynamic stress [11–13], and (5) co-culture with the accompanying cells of different types [5, 14]. Microfabrication techniques were also used for many of the *in vivo*-like cellular systems [15].

Lab-on-a-chip microsystems have found widespread applications in life sciences over the past decade [16–20]. The rising interest is determined mostly by advantages offered by microfluidic systems: low cost, portability, possibility of nanoliter sample handling *etc*. There are also a number of factors and phenomena possible to achieve only in the microenvironment: capillary forces, laminar flows, diffusion as the major means of mass transport, high surface area to volume ratio and precise control over liquid perfusion. The mentioned, unique properties of a lab-on-a-chip inner environment proved to be highly advantageous for cell engineering [21–23], and many solutions for *in vivo*-mimicking microfluidic systems could have been developed [15].

The aim of our project was to develop a novel integrated system for three-dimensional human cell culture and anticancer drug testing. The presented solution couples advantages of lab-on-a-chip systems with well known, but not widely used, three-dimensional cellular model: Multicellular Tumor Spheroid (MCTS). MCTS is considered as the best cellular model for anticancer therapy testing developed so far [4, 24]. MCTS presents morphology and physiology similar to

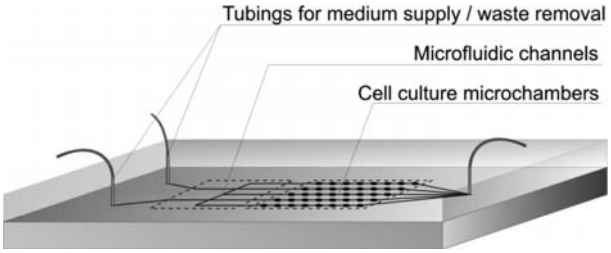

Fig. 1. Outlook of a lab-on-a-chip system for multicellular tumor spheroid culture.

tumor *in vivo* with the network of cell-cell interactions and junctions, presence of extracellular matrix, three--dimensional structure and nutrients, metabolites and oxygen gradients [25]. Widespread use of the MCTS model is constricted by cultivation limitations. A number of methods of MCTS formation were described in literature and several found their final applications [26]. However, most of them cause variation in size or are cost, labor and energy consuming [27, 28].

We present portable and easy to handle devices for MCTS formation, culture and analysis. Dimensions of MCTS (over 150 μm) exacted three-dimensional structure of the microfluidic system. Design of the microsystems consists of a matrix of microchambers for single spheroid cultivation connected with a network of microfluidic channels for media supply and waste removal (Fig. 1). Two systems were fabricated and tested and long-term culture of human colon carcinoma cells (HT-29) was performed.

Design and fabrication

Poly(dimethylsiloxane) (PDMS) was chosen as a material for fabrication of cell culture systems. PDMS exhibits a number of properties advantageous for cell culture devices: biocompatibility and lack of toxicity, transparency enabling observation, and gas permeability for culture oxygenation and buffering [29, 30]. The hydrophobicity of its surface prevents cell adhesion and therefore promotes spontaneous aggregation and spheroid formation. Moreover, replica molding technique provides fast and cheap prototyping of microstructures in PDMS with a submicron resolution [31, 32]. Two different techniques of microfabrication in PDMS were applied for MCTS culture microdevices manufacturing.

Photolithography and replica molding

Among the techniques of micromachining of a master for PDMS replica molding, photolithography is the most widely used [33]. Our group optimized the technology of master fabrication by photolithography using a capillary film [16]. This technique provides fabrication of two-dimensional structures of uniform depth (corresponding with a thickness of a photoresist). The capillary film used for cell culture device fabrication was 50 μm thick so the multi-layered system was required for achievement of a structure compatible with MCTS dimensions. Three-dimensional structure was obtained by combining of three layers of two-dimensional PDMS structures (Fig. 2).

First, geometry of the structure was designed using AutoCAD software. A photomask was obtained by high--resolution print on the transparent foil. Then a photoresist (a capillary film) was placed on a glass wafer (Fig. 2: 1) and exposed to the UV light through the photomask (Fig 2: 2). Unexposed areas of the photoresist were rinsed away and a master was obtained (Fig. 2: 3). PDMS pre-polymer was mixed with the curing agent in 10:1 weight ratio. Liquid mixture was poured over the

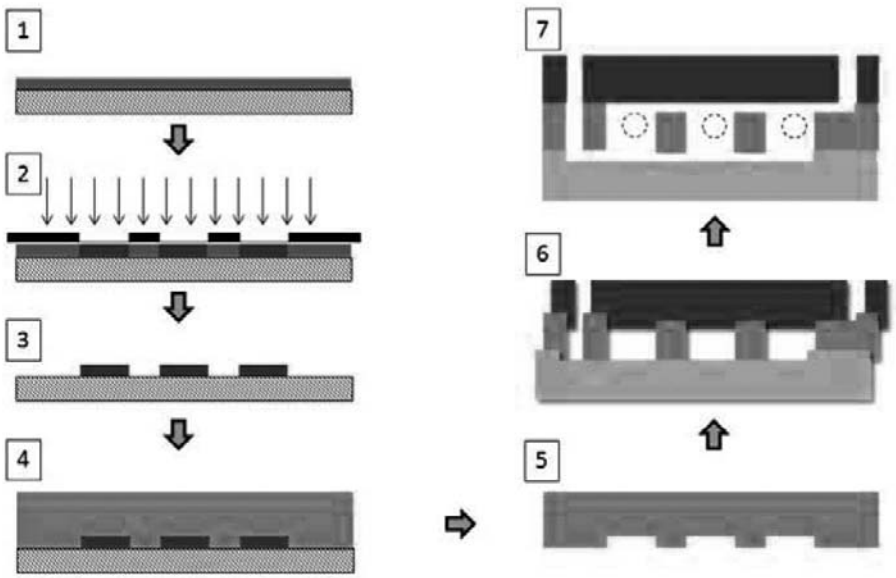

Fig. 2. Fabrication of a multi-layered microsystem using photolithography and replica molding — see text for details.

master and left for cross-linking for 2 hours in 70°C (Fig. 2: 4). PDMS was peeled off from the master and a 50 μm deep microstructure was obtained (Fig. 2: 5). Elements of PDMS, frozen in liquid nitrogen were drilled through to obtain cell culture microchambers and inlets and outlets (Fig. 2: 6). Elements of the microsystem were adjusted and bonded using oxygen plasma treatment. Three-dimensional structure was obtained by two step bonding of three layers of PDMS (Fig. 2: 7; dashed circles correspond to spheroid cultured in the microchambers). Finally, an array of perfusion microchambers 700 μm wide and 1mm deep, and network of channels 300 μm wide and 50 μm deep was obtained ("Sphero I" microsystem).

Double casting using thermal aging

Multi-layered three-dimensional PDMS systems meet some limitations. First of all, dimensions of such structures are dependent on photoresist or PDMS layer thickness. On the other hand, manual adjustment of many layers is problematic and mismatching can lead to perfusion disturbances. Therefore other techniques of fabrication of three-dimensional PDMS structures are required.

Micromilling is a technique suitable for fast three--dimensional microfabrication in thermoplastic materials (poly(methyl methacrylate), polycarbonate etc.) [34]. Unfortunately, it is not suitable for fabrication in PDMS. To fabricate a copy of micromilled poly(methyl methacrylate) (PMMA) structure, double casting of PDMS was utilized. Double casting of PDMS is a two-step process, where first PDMS replica is a master for second replication step (Fig. 3). Due to strong adhesion and partial cross-linking between PDMS master and PDMS cast, demolding led to the microstructure damage (Fig. 4). The possible solution for this problem is covering of a PDMS master with hydrophilic polymers [35–37]. However, chemical modification can effect the microstructure geometry and may influence biocompatibility of the device [38]. We developed an alternative, low-cost, non-chemical method of enhancing efficiency of double casting prototyping by thermal aging of PDMS master. Thermal aging, also known as extended curing, leads to crosslinking of low molecular weight chains remained in a PDMS bulk and changes its surface from hydrophobic to more hydrophilic [39].

First, the geometry of the structure was designed using AutoCAD software and micromilled in a PMMA plate (Fig. 3: 1, 2). Then, standard PDMS replica molding was performed (Fig. 3: 3, 4) and a convex PDMS microstructure was obtained. The PDMS replica was thermally aged in a laboratory dryer set for 100°C for 48 hours (Fig. 3: 5). Thermally modified PDMS structure was a master for second replica molding (Fig. 3: 6, 7). After second replication, an accurate copy of a PMMA structure was obtained (Fig. 4). Three--dimensional PDMS structure was bonded with another

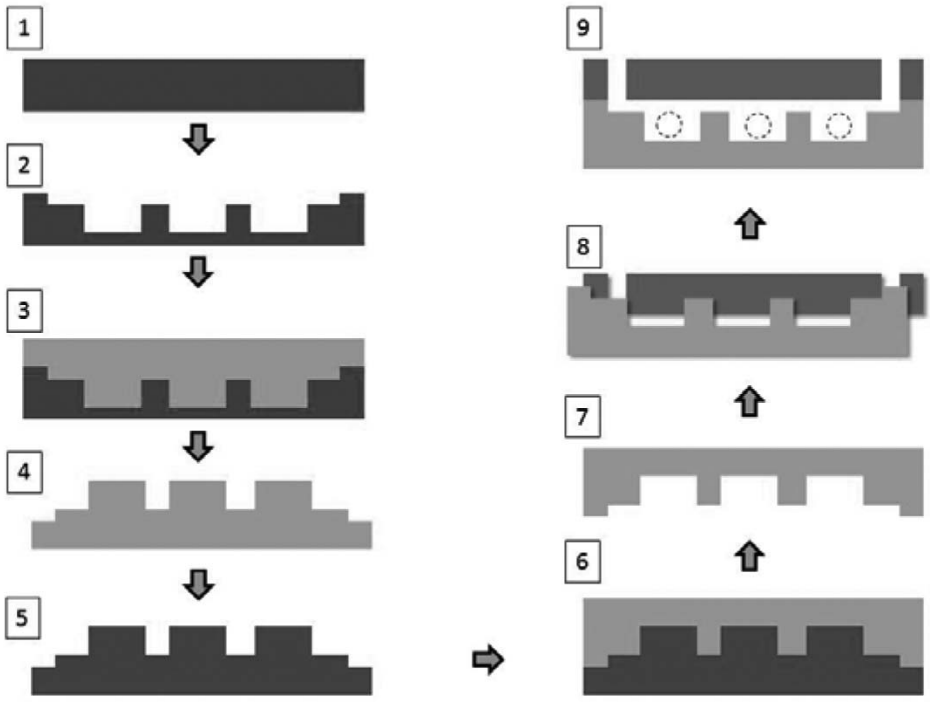

Fig. 3. Fabrication of a three-dimensional structure in PDMS using micromilling and double casting with thermal aging step — see text for details.

PDMS plate, and a microfluidic system was obtained (Fig. 3: 8, 9). Final device consisted of 1 mm and 50 μm deep wide microchannels and 200 μm wide and 200 μm deep cell culture microwells ("Sphero II" microsystem).

During our investigations optimal conditions for effective fabrication were found. Stiffness of PDMS was modified by its composition: 8:1, 9:1 and 10:1 pre-polymer to curing agent weight ratios were considered. 8:1 PDMS was rigid but breakable and 10:1 PDMS was too soft and elastic. Therefore 9:1 PDMS was chosen for the fabrication of both: master and replica. Time of thermal aging was also optimized. PDMS masters were exposed to thermal aging (100°C) for 24, 48 and 72 hours. Hydrophobicity and accuracy of replication were measured for those masters. It was found that after 48 hours of aging contact angle of a water drop on PDMS surface changes from 97° to 85°, which means that it turns more hydrophilic. This modification was efficient enough for successful replication (Fig. 4). 9:1 PDMS master aged for 48 hours can be used for at least three sequencing replications.

Cell culture

Petri dish spheroid culture

The biocompatibility of PDMS surface was verified by cell culture on PDMS-covered plates. 35mm polystyrene Petri dishes were covered with thin layer of PDMS and left for cross-linking under dust-free conditions. Next, plates and covers were exposed to UV light for 20 minutes for sterilization, closed and sealed with a Parafilm foil.

HT-29 human colon carcinoma cells were suspended in RPMI medium supplemented with 20% of fetal bovine serum (FBS). Approximately $2 \cdot 10^5$ cells in 2 mL of medium were seeded on each plate. Culture was

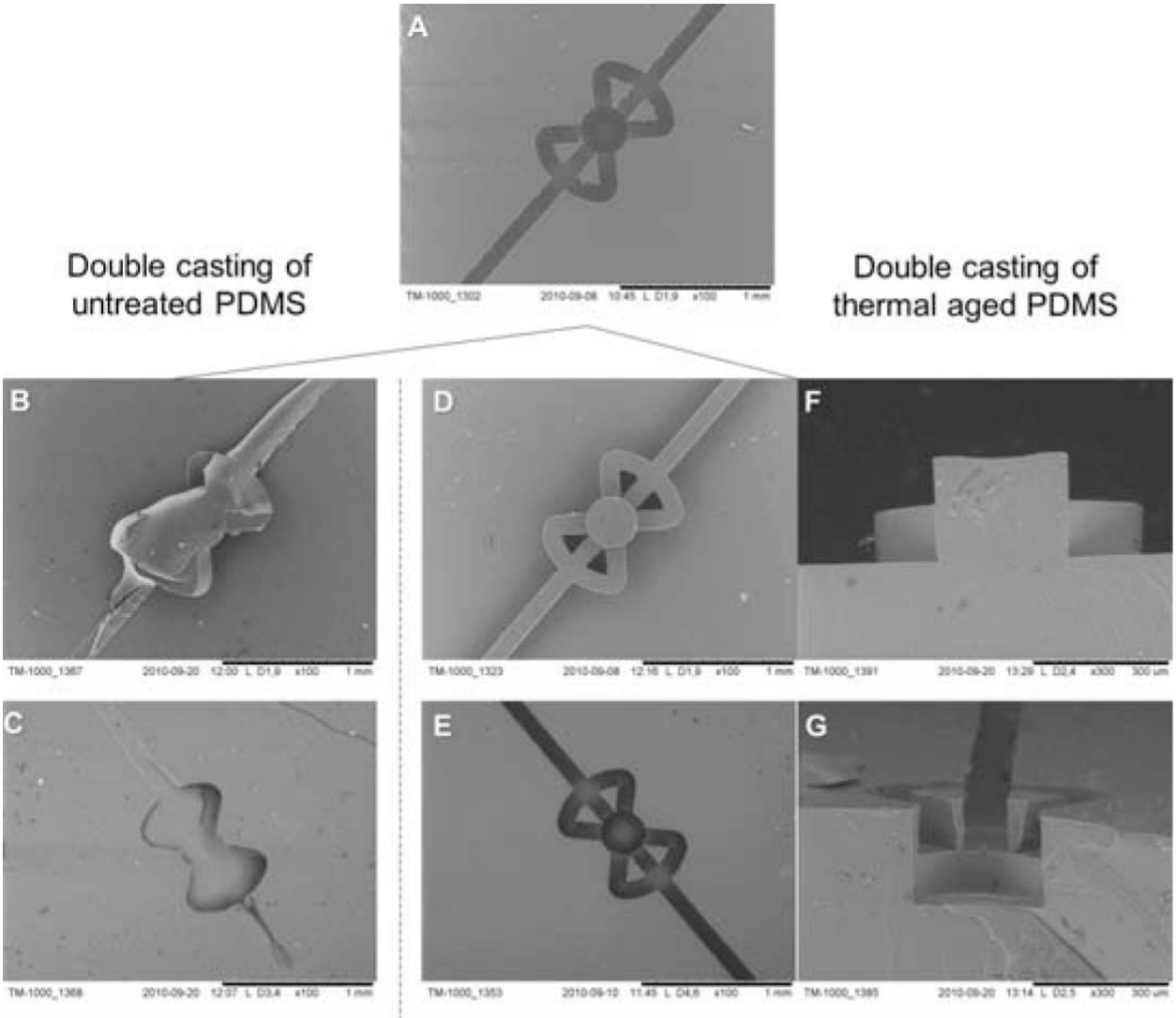

Fig. 4. Verification of accuracy of replication using scanning electron microscope: (A) microstructure micromilled in PMMA; (B) damaged untreated PDMS master and (C) replica; (D, F) thermally aged PDMS master used for replication and (E, G) PDMS replica. Structures B–G were made of 9:1 PDMS.

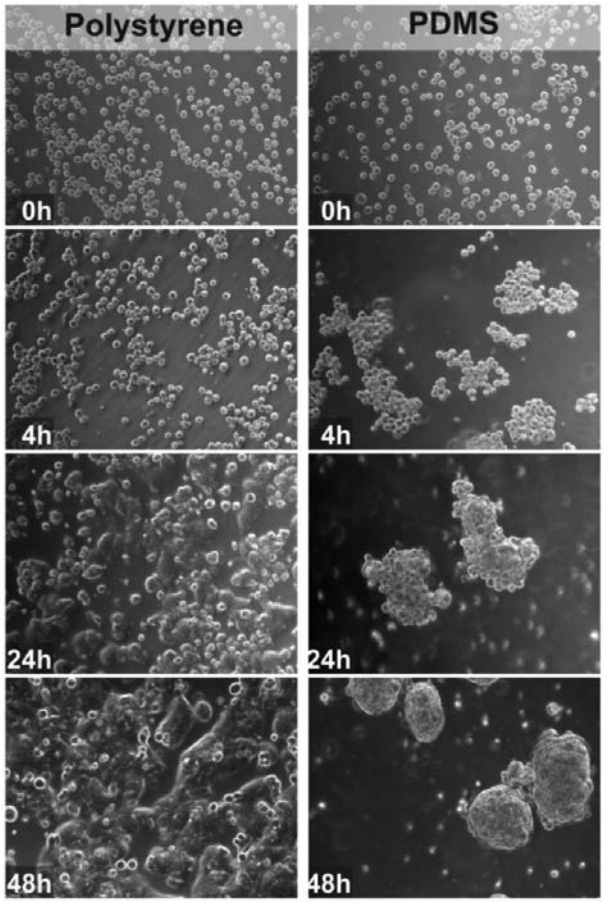

Fig. 5. Culture of HT-29 cells on two substrates: polystyrene and poly(dimethylsiloxane) (PDMS).

carried both on non-adhesive PDMS-covered plates and strongly adhesive polystyrene plates.

Results of the culture are presented in Fig. 5. There are significant differences in affinity between cells and substrates, visible within the first hours of the culture. Cell cycle of HT-29 cells depends on the adhesive proteins (i.e. integrin). Therefore possibility of adhesion is essential for cell growth. Surface of polystyrene promotes adhesion of individual cells and monolayer is created during following hours of the culture. Adhesive proteins are bonded to the artificial polymer.

Cells cultured on the PDMS surface act in a different way. Hydrophobicity of the surface prevents cell adhesion. Therefore cells aggregate during first hours of culture *via* integrin interactions. Then, proteins of extracellular matrix (ECM) are secreted and form a natural scaffold for cell adhesion. During next hours cadherin expression is observed and compact spheroids are formed (24 h). Further cell growth is dependent on proliferative layer of the spheroids. The spheroid model closely mimics the *in vivo* environment, because of the three-dimensional architecture of tissue and presence of ECM.

Experiments of Petri dish culture of spheroids also revealed disadvantages of this type of culture. First of all, there is no possibility of medium exchange during culture. While monolayer cultured cells stay adhered to the culture dish during medium exchange, it is not possible for suspended spheroids. Attempts of centrifugation at different parameters have been performed, however hydrodynamic stress led to spheroids' disintegration. Therefore, only medium refill is possible, but accumulation of metabolites disables culture longer than 5 days. Another issue, which is problematic, is monitoring of cell growth. There is no possibility of single spheroid observation. Also connecting of spheroids disables estimation of a growth rate. Our microsystems are potential solutions to the mentioned issues.

Microsystem spheroid culture

Fabricated microsystems were sterilized by exposition to UV light (20 min) and following perfusion of 70% ethyl alcohol (30 µL/min, 20 minutes). Next, they were filled with RPMI medium supplemented with 20% FBS and left in the CO_2 incubator (37°C) for an hour. HT-29 cell suspension ($1 \cdot 10^6$ cells/mL) was introduced into each microsystem at a maximum flow rate of 20 µL/min. Microsystems were sealed and placed into the incubator for culture.

Spheroid culture in the microfluidic systems consisted of four stages (Fig. 6): (I) seeding, (II) aggregation, (III) unaggregated cells' removal and (IV) spheroid formation. First, cells were introduced to the whole volume of the microsystem. Within first 16 hours of incubation cells

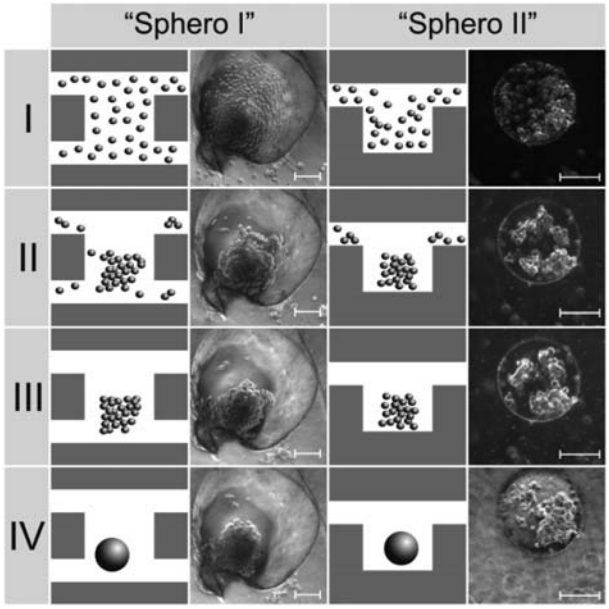

Fig. 6. HT-29 spheroid culture in two types of microsystems: (I) cell seeding, (II) cell aggregation after 16 hours of culture, (III) medium exchange washing away unaggregated cells and (IV) spheroid formation within following 24 hours. Scale bars stand for 100 μm.

aggregated, because microsystem's building material (PDMS) prevents cell adhesion. Then medium perfusion was applied for two reasons: to remove unaggregated cells and exchange medium to a fresh one. The flow rate was selected to effectively remove cells from microchannels and minimize hydrodynamic stress affecting remained cells. Within next 24 hours cells remained in the microchambers formed spheroids. Medium was exchanged every 48 hours during culture.

HT-29 multicellular tumor spheroid formation and cultivation using developed microfluidic systems were very effective. Spheroids were cultured for over two weeks in both types of the devices. Medium exchange every second day provided nutrients supply and metabolites removal, which is a significant advantage over a batch, Petri dish MCTS culture. Another important issue is a possibility of single spheroid observation. Construction of both devices disables translocation of spheroids exceeding 50 μm. In practice — it means that all aggregated cells remain in the initial microchamber/microwell for the whole period of culture. Therefore direct observation of spheroid growth was possible. It was observed that after 7–10 days of culture spheroids' diameters reached 200–250 μm in "Sphero I" microsystem and 150–200 μm in "Sphero II" microsystem. Then, a growth rate slowdown was observed. It is expected that this slowdown is dependent on nutrient and oxygen diffusion limitations. Spheroids reached their maximum diameters and this state was maintained for the rest of the culture. High viability was confirmed at the endpoint of every culture by the viability assay using Calcein-AM and Propidium Iodide fluorescent dyes. Lack of proliferation and stable viability may be symptoms of homeostasis-like state achievement in the microenvironment.

Perspectives

Developed lab-on-a-chip systems proved to be highly advantageous for three-dimensional carcinoma cell culture. This is a prospective solution for cancer research and anticancer drug screening. Cell culture microarray can be easily coupled with other microfluidic geometries such as concentration gradient generator [29]. It is a solution for automation of the assays and a step towards high-throughput screening using three-dimensional cell culture.

Another challenge is quantitative data obtainment from three-dimensional culture. There are a lot of assays and techniques for monolayer cultured cells' analysis. Unfortunately, few are compatible with the spheroid model [26]. Our efforts are now focused on development and adaptation of analytical methods for multicellular spheroid testing.

Conclusions

In this paper, two novel devices for three-dimensional cell culture were presented. The geometries of perfusion microchambers and microwells were tested. During the investigations, a novel technology of microfabrication was developed and successfully applied. HT-29 cells were cultured as multicellular tumor spheroids for over two weeks and high viability was observed within this time.

Acknowledgements

This work has been supported by Polish Ministry of Science and Higher Education through "Iuventus Plus" Programme (2011).

References

[1] Cukierman, E., R. Pankov, and K. Yamada. "Cell interactions with three-dimensional matrices." *Current Opinion in Cell Biology* 14 (2002): 633–639.

[2] Yamada, K., and E. Cukierman. "Modeling tissue morphogenesis and Cancer in 3D." *Cell* 130 (2007): 601–610.

[3] Griffith, L., and M. Swartz. "Capturing complex 3D tissue physiology *in vitro*." *Nature Reviews Molecular Cell Biology* 7 (2006): 211–224.

[4] Lin, R., and H. Chang. "Recent advances in three--dimensional multicellular spheroid culture for biomedical research." *Biotechnology Journal* 3 (2008): 1172–1184.

[5] Guillouzo, A., and C. Guguen-Guillouzo. "Evolving concepts in liver tissue modelling and implications for *in vitro* toxicology." *Expert Opinion on Drug Metabolism and Toxicology* 4 (2008): 1279–1294.

[6] Desai, T. "Micro- and nanoscale structures for tissue engineering constructs." *Medical Engineering &. Physics* 22 (2000): 595–606.

[7] Fischbach, C., et al. "Engineering tumors with 3D scaffolds." *Nature Methods* 4 (2007): 855–860.

[8] Friedrich, J., R. Ebner, and L. Kunz-Schughart. "Experimental anti-tumor therapy in 3-D: Spheroids — old hat or new challenge?" *International Journal of Radiation Biology* 83 (2007): 849–871.

[9] Kelm, J., et al. "Method for Generation of Homogenous Multicellular Tumor Spheroid Applicable to a Wide Variety of Cell Types." *Biotechnology and Bioengineering* 83 (2003): 173–180.

[10] Borenstein, J., et al. "Microfabrication Technology for Vascularized Tissue Engineering." *Biomedical Microdevices* 4 (2002): 167–175.

[11] Cunningham, K., and A. Gotlieb "The role of shear stress in the pathogenesis of atherosclerosis." *Laboratory Investigation* 85 (2005): 9–23.

[12] Blackman, B., G. Garcia-Cardena, and M. Gimbrone. "A New *In Vitro* Model to Evaluate Differential Responses of Endothelial Cells to Simulated Arterial Shear Stress Waveforms" *Journal of Biomechanical Engineering* 124 (2002): 397–407.

[13] Keane, J., D. Ryan, and P. Gray. "Effect of Shear Stress on Expression of a Recombinant Protein by Chinese Hamster Ovary Cells." *Biotechnology and Bioengineering* 81 (2003): 211–220.

[14] Seo, S., et al. "Enhanced liver functions of hepatocytes cocultured with NIH 3T3 in the alginate/galactosylated chitosan scaffold." *Biomaterials* 27 (2006): 1487–1495.

[15] Ziółkowska, K., R. Kwapiszewski, and Z. Brzózka. "Microfluidic devices as tools for mimicking the *in vivo* environment." *New Journal of Chemistry* 35 (2011): 979--990.

[16] Chudy, M., et al. "Miniaturized tools and devices for bioanalytical applications: an overview." *Analytical and Bioanalytical Chemistry* 395 (2009): 647-668.

[17] Andersson, H., and A. Van Den Berg. "Microfabrication and microfluidics for tissue engineering: state of the art and future opportunities." *Lab on a Chip* 4 (2004): 98–103.

[18] Bhadriraju, K., and C. Chen "Engineering cellular microenvironments to improve cell-based drug testing." *Drug Discovery Today* 7 (2002): 612–620.

[19] Park, T., and M. Shuler. "Integration of Cell Culture and Microfabrication Technology." *Biotechnology Progress* 19 (2003): 243–253.

[20] Shim, J., et al. "Micro- and Nanotechnologies for Studying Cellular Function." *Current topics in medicinal chemistry* 3 (2003): 687–703.

[21] Leclerc, E., Y. Sakai, and T. Fujii. "Cell Culture in 3-Dimensional Microfluidic Structure of PDMS (polydimethylsiloxane)." *Biotechnology Progress* 20 (2004): 750–755.

[22] Walker, G., H. Zeringue, and D. Beebe. "Microenvironment design considerations for cellular scale studies". *Lab on a Chip* 4 (2004): 91–97.

[23] Chen, C., et al. "Antimicrobial susceptibility testing using high surface-to-volume ratio microchannels." *Analytical Chemistry* 82 (2010): 1012–1019.

[24] Poland, J., et al. "Comparison of protein expression profiles between monolayer and spheroid cell culture of HT-29 cells revealed fragmentation of CK18 in three--dimensional cell culture." *Electrophoresis* 23 (2002): 1174–1184.

[25] Sutherland, R. "Cell and Environment Interactions in Tumor Microregions: The Multicell Spheroid Model." *Science* 240 (1988): 177–184.

[26] Pampaloni, F., E. Stelzer, and A. Masoti. "Three-Dimensional Tissue Models for Drug Discovery and Toxicology." *Recent Patents on Biotechnology* 3 (2009): 103–117.

[27] Ziółkowska, K., et al. "Lab-on-a-chip for cell engineering: towards cellular models mimicking *in vivo*." *Challenges of Modern Technology* 1.2 (2011): 79–82.

[28] Torisawa, Y., et al. "A multicellular spheroid array to realize spheroid formation, culture, and viability assay on a chip." *Biomaterials* 28 (2007): 559–566.

[29] Ziółkowska, K., et al. "PDMS/Glass microfluidic cell culture system for cytotoxicity tests and cells passage." *Sensors and Actuators B* 145 (2010): 533–542.

[30] Velve-Casquillas, G., et al. "Microfluidic tools for cell biological research." *Nano Today* 5 (2010): 28–47.

[31] McDonald, J., and G. Whitesides. "Poly(dimethylsiloxane) as a Material for Fabricating Microfluidic Devices." *Accounts of Chemical Research* 35 (2002): 491–499.

[32] Regehr, K., et al. "Biological implications of polydimethylsiloxane-based cell culture." *Lab on a Chip* 9 (2009): 2132–2139.

[33] McDonald, J., et al. "Fabrication of microfluidic systems in poly(dimethylsiloxane)." *Electrophoresis* 21 (2000): 27--40.

[34] Becker, H., and C. Gärtner. "Polymer microfabrication technologies for microfluidic systems." *Analytical and Bioanalytical Chemistry* 390 (2008): 89–111.

[35] Wong, I., and C. Ho. "Surface molecular property modifications for poly(dimethylsiloxane) (PDMS) based microfluidic devices." *Microfluidics and Nanofluidics* 7 (2009): 291–306.

[36] Briones, M., et al. "Practical Method for Rapid Microchannel Fabrication in Polydimethylsiloxane by Replica Molding without Using Silicon Photoresist." *Journal of Chemical Engineering of Japan* 39 (2006): 1108–1114.

[37] Koerner, T., et al. "Epoxy resins as stamps for hot embossing of microstructures and microfluidic channels." *Sensors and Actuators B* 107 (2005): 632–639.

[38] Gitlin, L., P. Schulze, and D. Belder. "Rapid replication of master structures by double casting with PDMS." *Lab on a Chip* 9 (2009): 3000–3002.

[39] Eddington, D., J. Puccinelli, and D. Beebe. "Thermal aging and reduced hydrophobic recovery of polydimethylsiloxane." *Sensors and Actuators B* 114 (2006): 170–172.