An integrated gas removing system for microfluidic application. Design and evaluation

Iga Martyna Hofman, Karina Ziółkowska, Artur Dybko, Zbigniew Brzózka
Faculty of Chemistry, Warsaw University of Technology, Warsaw, Noakowskiego 3 Street.
e-mail: iga.hofman@gmail.com

Microfluidic systems are used in a wide range of applications, including medical diagnostics, cell engineering and bioanalytics. In this work we focused on "*Lab-on-a-chip*" microsystems for cell cultivation. A troublesome problem of gas bubbles entering microdevices causing signal interferences and cells damage was emphasized. A novel, integrated debubbler in the form of cylindrical traps covered with thin PDMS membrane was designed and manufactured. Demonstrated debubbler was successfully applied in a long-lasting culture of HT-29 cell aggregates.

Keywords and phrases: gas removing system, debubbler, debubbling system, lab-on-a-chip, microfluidics, long-term cell culture, cell spheroids, HT-29 cells.

Introduction

Still growing demand for fast, cheap, reliable and reagent-reduction laboratory methods has recently led to rapid development of microtechnology. Miniaturization covers many scientific and technical branches as electronics, (bio)analytical chemistry, medical diagnostics or cell engineering [1-4]. Microfluidics studies behaviour of fluids in systems of very small dimensions and volumes (of the order of magnitude of micro-, nano- or even picolitres). *Lab-on-a-chip* microsystems are devices that integrate few different modules onto one single chip. That enables conducting complete laboratory process, from probe sampling to signal measurement, using one device only [5]. Due to its unique properties *Lab-on-a-chip* systems are applied in such biomedical assays as cell and blood analysis, cytometry, nucleic acids amplification, genetic mapping, enzymatic assays, protein separation, toxicity analysis and drug discovery [6]. In studies using biosamples sterility is extremely important, what in case of sealed, primarily sterilized systems doesn't bring inconvenience. Moreover, volumes of available biosamples are often limited (sometimes micro- or even nanolitres) and insufficient to analyse in a macroscale [6].

Apart from many facilities (including laminar flow, advantageous surface–area–to–volume ratio or increased sensitivity) that using microdevices brings, some difficulties also occur. Gas bubbles penetrating microchannels are one of them. The problem is observed in many different types of microsystems, nevertheless, in case of cell cultures gas bubbles are particularly undesirable. Not only do they cause flow and signal interferences, but also damage cells mechanically. What is more, when culture medium is replaced with a gas bubble, cells are devoid of nutrients and growth factors, pH of their environment is not stabilized in any way, which may eventually lead to cells death.

In this work we focused on designing and developing an integrated debubbling system for variety of microfluidic application. The aim of the project was to develop a novel, cheap, efficient and simple solution, which could be an integral part of a main microsystem. To prove its utility, gas removing system was applied in a long-lasting cell culture of HT-29 aggregates.

Design and fabrication

Fabrication method of microsystems depends on used material as well as channels structure and geometry. Most common construction materials are polymers (PDMS, PMMA, PS) (Fig. 1), glass and silicon [5].

Complete manufacture process consists of few steps: microchannels patterning, channels sealing and additional elements joining. Depending on available equipment and desired application of a device, a proper material is chosen.

Microsystems for cells cultivation and debubbler testing were made of poly(dimethylosiloxane). PDMS is the most frequently applied material due to its advantageous properties. PDMS is easily formed elastomer, transparent in a visible electromagnetic spectrum [9-10], which enables microscopic observation. It is also gas permeable, non-toxic and biocompatible polymer, which is especially important while handling any biological samples [11]. Another important issue is the fact that fabrication methods of PDMS require neither extremely high temperatures or voltage nor ultra clean conditions [5]. Micromachining methods include replica molding and double casting [12].

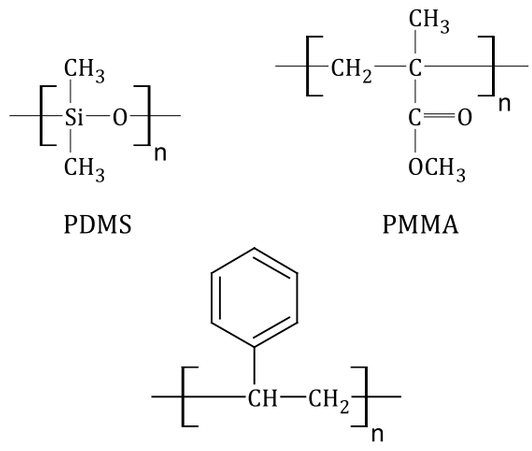

Figure 1. Structural formula of polymers most frequently used for microsystems fabrication

Cells culture microfluidic system integrated with gas removing module

Geometry of the microsystem (Fig. 2) was designed using AutoCAD software. Because of the 3D structure of the system, photolithography was not suitable for preparing the mould. Instead, micromiling combined with double casting [13] was applied. Firstly, designed pattern was micromilled in a PMMA wafer, which was then used to produce first PDMS replica. After 72-hour thermal aging in 100°C, first replica served as a master for second PDMS replica, which was an exact copy of the PMMA structure.

Direct micromilling in PDMS layer wouldn't be possible because this method is only suitable for thermoplastic materials as PMMA or PC [13].

After cross-linking, PDMS layer with embedded microchannels was sealed with another plain PDMS plate with drilled inlet, outlet and debubbling microwells. Bonding was preceded by oxygen plasma activation of surfaces. Last step was to cover debubbling wells with thin PDMS membranes also after plasma activation. Membranes were prepared by pouring a thin layer (about 0,5 mm) of liquid PDMS, which after cross-linking was cut into rectangles, on a plate. Inlets, outlets and debbubling holes were made in a plain layer with 1,3 millimeter drill.

Thickness of each PDMS layer equaled 0,4 cm, length 5 cm and width 1,6 cm. Microchannels diameter was 1 mm and cell culture microwells diameter was 200 µm.

Geometry of the cell culture system was design by Ziółkowska *et al* . [14].

Time duration and temperature of the cross-linking process were optimized by CSRG Group. Also proper weight ratio of PDMS prepolimer to curing agent used in double casting method was estimated [13]. Ratio 9:1 resulted in polymer of the best stiffness and flexibility, which enabled PDMS master to be utilized even 3 times without any damage of structure or cross-linking with another liquid layer of the polymer [15].

Microsystems for debubbler testing

Gas removing module was integrated with number of microsystems of different channel geometries. Microsystems were constructed by photolithography and replica moulding techniques. First step was to design required structures in AutoCAD software. Then masks were printed on a transparent foil. Masks were utilized in a photolithographic process of creating masters for replica moulding.

A novel method in which capillary film replaced SU-8 photoresist was optimized by CSRG group [12]. A piece of capillary film of 50 µm thick was attached to square plexi glass plate, then through a photomask exposed to UV light for 180 s. Unexposed area of the photoresist was rinsed out with water. Created pattern was dried with compressed nitrogen. In the next step liquid mixture of PDMS pre-polymer and curing agent (weight ratio 10:1) was poured into the mould with the master in the bottom and left for 2 hours in 70°C for cross-linking. Plain layer of microsystem was made in the same way, the only difference was lack of patterned master in the bottom of the square mould. After immersing in liquid nitrogen, inlets, outlets and debubbling wells were drilled in the plain cross-linked plate of PDMS. Two layers of microsystem were bond together using oxygen plasma activation. In the figure 3 a complete microsystem is presented.

Gas removing system evaluation

During investigation influence of channels width, flow rates and wells position on the debubbler efficiency was evaluated.

Firstly, wells were placed over channels of various widths, from 100 µm to 1 mm. Fluid was let through the devices with gradually increasing flow rates (in the range of 5-100 µl/min). After every change of flow rate, an air bubble was let into the system through the inlet tubing.

Presented microsystem operates as follows: an air bubble, which enters the system through the inlet, moves along the channel till it encounters the well. Then, because its density is lower than liquid density, bubble is pushed

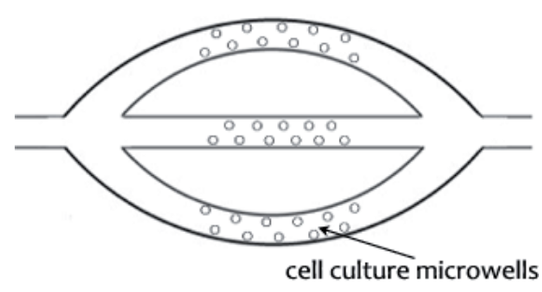

Figure 2. Geometry of microsystem for cell cultivation

up and trapped under the membrane. Liquid flows continuously to the outlet. The process of an air bubble trapping is shown in the figure 5.

It was proved that diameter of well to channel width ratio is of vital importance. If well diameter is too small resistance to motion upward the well can be greater than resistance to motion along the channel, which results in omitting the well by the air bubble. With channel width equal to 100 µm, well diameter (1,2 mm) to channel width ratio is 12. Air bubble is trapped in the well even with flow rate equal to 100 µl/min. While channel width increases, the ratio decreases and efficiency of debubbling module deteriorates. When channel width equals 200 µm, debubbler functions correctly till flow rate 30 µl/min, with channel width 300 µm (and more) system is inefficient even at flow rate as low as 5 µl/min.

In order to enable proper functioning of debubbler in systems with desired channels over 300 µm wide, wider channels were preceded by thinner ones (100 µm) over which wells were positioned. A number of geometries were designed. All of them are presented in the figure 6.

First option (Fig. 6a.) with narrowing part of a channel proved inefficient. It is most likely caused by increasing speed of an air bubble when flowing from wider to thinner part of a channel (from Bernoulli's law – the smaller channel cross-section the higher speed of a fluid and lower pressure), which hampers trapping air bubble in the well.

Elongating thin channel (Fig. 6b, c) likewise dividing wider channel into three thinner ones (Fig. 6d), improved system performance. Spiral and meandered geometry were proposed due to limited length of a whole microsystem.

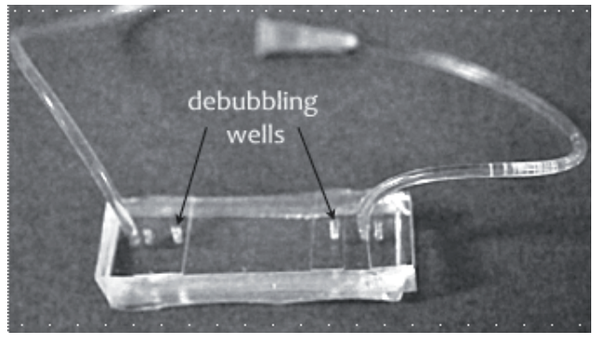

Figure 3. A complete PDMS microsystem with integrated debubbler

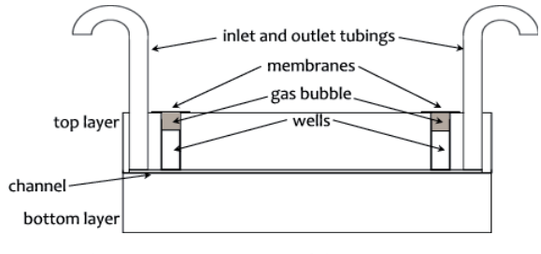

Figure 4. Schematic cross-section of the microsystem

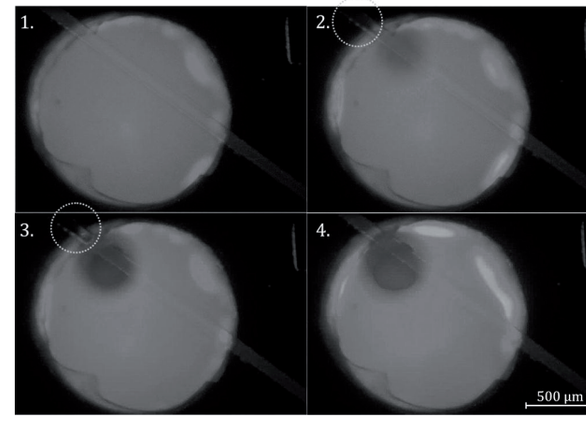

Figure 5. Process of system testing; flow rate = 100 µl/min. 1 – only liquid flows along the channel, 2 – air bubble enters the system, 3 – air bubble penetrates the well, visible gap in a liquid flow before the well, 4 – bubble trapped in the well, no interferences in liquid flow after the well

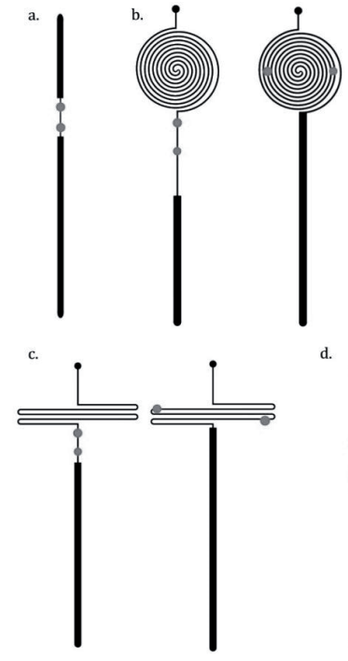

Figure 6. Different geometries of microchannels; dots represent debubbling wells

Amount of air that can be trapped in the well is constrained by its volume. In order to increase module capacity few wells – one after another – can be applied.

Cells cultivation

Cell engineering investigates influence of different factors on cells proliferation. It also formulates new drugs and active substances.

That is why developing an appropriate model accurately imitating *In vivo* conditions is of vital importance [16].

However, the task is not so simple since organs' structure is complex and many interactions occur between them. What is more, a simultaneous influence of a substance on a whole organism should be taken into consideration [17-19].

Cell culture microsystems

Cell culture microsystems provide many advantages over traditional cell cultures in a macroscale. First and foremost, in microsystems better control over cells' microenvironment and their mutual interactions can be achieved. Moreover, microfluidic systems allow continuous delivery of nutrients and adjusting flow rates so that the shear stress corresponds to physiological conditions [20-21].

Small dimensions of microchannels provide laminar flow of liquid, which results in a lack of turbulence. Owing to that mass transport, in a direction perpendicular to motion direction, is possible only by diffusion, analogically to *In vivo* conditions [22]. Beneficial surface area to volume ratio, typical for microsystems, provides cells with more effective oxygen supply [23].

Much less reagents consumption, which results from low systems volumes, is also undeniable advantage.

Conditions of cell cultures

Natural, biochemical environment, in which cells grow and proliferate, is composed of cytokines, growth factors, hormones and other substances that participate in signal transmitting and regulate cells' evolution [24]. In *In vitro* conditions cells are immersed in a culture medium, which should contain all essential components. Main of them are nutrients and growth factors – amino acids, glucose, vitamins and other inorganic substances like mineral salts ($CaCl_2$, KCl, $MgSO_4$, NaCl, NaH_2PO_4) [24]. Environment should also be stabilized, especially important is maintaining constant pH value, which is most favorable for cell proliferation. Therefore, buffers, usually bicarbonate buffering system, are applied. To prevent bacterial growth, antibiotics (e. g. streptomycin, penicillin) are added to medium. Substantial parameters are also appropriate temperature of cell culture, oxygen availability and concentration of CO_2 in the atmosphere [7].

Types of cell cultures

There are many types of cell cultures. The most popular one is monolayer cell culture [24]. Adherent cells create monolayer by attaching to substrate via proteins or peptides adsorbed from culture media or proteins pre-immobilized on substrate [25-27]. Despite simplicity and common usage, this type of cell culture is not the best imitation of *In vivo* conditions, where network of chemical and spatial interactions between cells occurs. Cultivating cells on a flat layers leads to change in their morphology, physiology and gene expression [17-19, 28].

Other cell culture models, which better correspond with actual conditions present in live organisms, were proposed.

One of the solutions is cultivating cells in 3D structures [29]. The earliest model described is multicellular spheroids model [30]. Cells don't grow on a surface but form spherical aggregates by attaching to each other. That model has many features specific for physiological conditions, i. a. mutual interactions of cells and gradient of oxygen, nutrients and metabolites comparable to this present in a live tissue. This type of culture is particularly useful in cancer tumors examination [31,32].

Dynamic, tridimensional environment of a tissue in *In vitro* conditions is mimicked by porous microscaffolds constructed from polymers [19, 33], e. g. biodegradable copolymer poly(DL-lactide-co-glycolide) (PLGA), or poly (glycerol-sebacate) (PGS) or proteins.

Another possibility is encapsulation and immobilization of cells in hydrogels (e.g. cationic collagen or anionic terpolymer 2-hydroxyethyl methacrylatemethacrylic acid-methyl methacrylate (HEMA-MMA-MAA)) packed inside the microchannels [34].

Cells seeding

In our research HT-29 human colon carcinoma cells in the form of spherical aggregates were cultured.

Prior to cells seeding, microsystem was sterilized. 3 ml of 70% ethanol was perfused through the system using syringe pump. System was being simultaneously exposed to UV light.

After sterilization, system was filled with RPMI culturing media containing 20% of FBS. Then cells suspension was introduced to the system with syringe pump. Cell seeding flow rate equaled 10 µl/min. After 10 minutes inlet and outlet tubings were plugged with clogged needles filled with PDMS and microsystem was placed in the incubator (37°C, 5% CO_2 in the atmosphere). Parameters – cell seeding flow rate and seeding duration – were determined experimentally so that enough number of cells could accumulate in cell culture microwells.

Cells culturing

HT-29 cells were cultured for four weeks. Culture medium was changed every second day. To provide whole medium replacement, 400 l of medium was perfused through the system every time. Flow rate equal to 8,5 l/min guaranteed lack of mechanical cell damage and optimal time duration of the process. During these four weeks cells were kept in the incubator, only for the time of the medium exchange were they placed on a heated microscope table.

Microsystem for cell cultivation was integrated with designed debubbling module. Geometry of the system is presented in the figure 7.

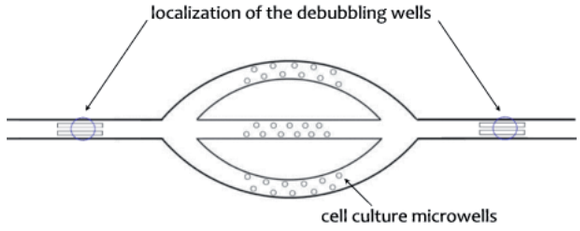

Figure 7. Geometry of the system for cell culturing integrated with debubbling module

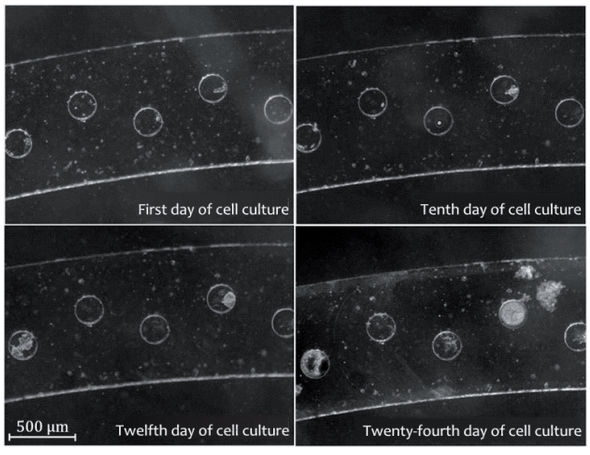

Figure 8. Sequential days of cell culture

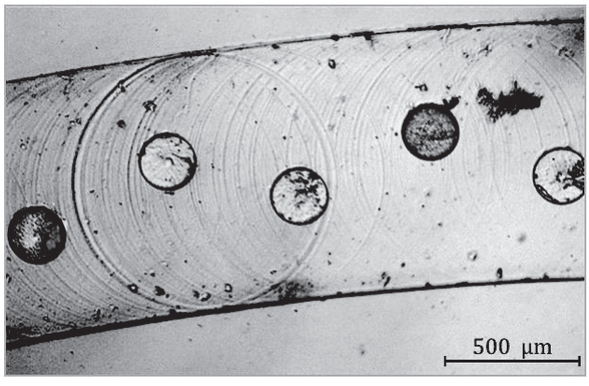

Figure 9. Result of a cellular viability test: live cells stained with calcein AM (green colour) and dead ones stained with propidium iodide (red colour)

Device consists of three channels in which culture microwells are grooved. The whole system is made of PDMS and is adapted to cultivating cells which compose spheroids and don't adhere to the hydrophobic surfaces. Utilization of the debubbler enabled long-lasting culture of HT-29 cell aggregates.

In the figure 8 sequential days of cell culture are presented.

After 4 weeks, a cellular viability test was conducted (Fig. 9 and 10). Cells were stained with two fluorescent dies – calcein AM and propidium iodide. In the UV light live cells, stained with calcein AM, emit green radiation, while dead ones, stained with propidium iodide, red radiation.

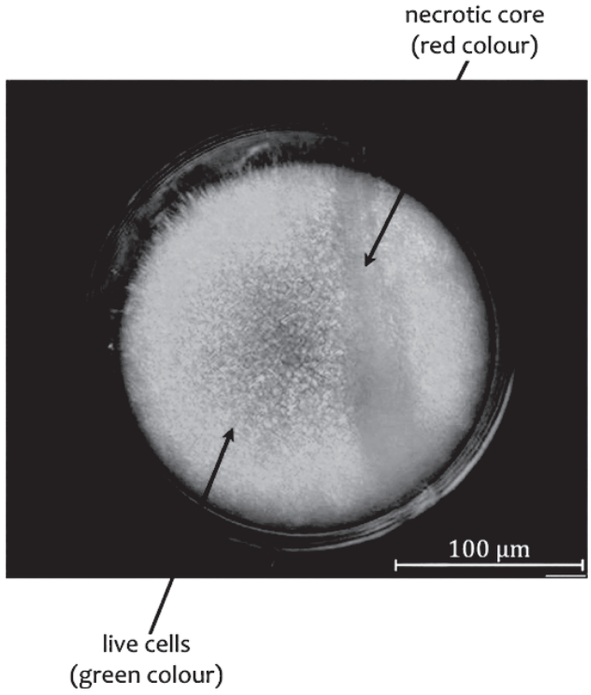

Figure 10. Cell spheroid stained with fluorescent dyes; characteristic necrotic core in the middle of the spheroid can be observed

Conclusions

Presented solution of gas removing system offers many advantages like simple and cheap fabrication process, no need of extra equipment enabling proper functioning, being an integrated part of a main microsystem of random geometry (which eliminates additional joints, inlets and outlets) and no liquid flow disorders. The influence of such parameters as flow rates and microchannels width on debubbler efficiency was tested. It was proved that well diameter to channel width ratio is of vital importance. The bigger the ratio is, the more efficiently debubbler function. Therefore, a number of geometries in which wells are positioned over the thin section of channel were designed. Presented geometries assure efficient performance of the module in a wide range of flow rates (5-100 µl/min). Utilization of the debubbling system enabled a cell culture of HT-29 aggregates, which lasted for four weeks.

Acknowledgements

This work was financially supported by Polish Ministry of Science and Higher Education through "Iuventus Plus" Programme, contract No. 0643/IP1/2011/71.

References

[1] Andersson, H., Van Den Berg A., *Lab Chip* 4 (2004): 98–103;
[2] Bhadriraju, K., Chen C., *Drug Discov Today* 7 (2002): 612–620;
[3] Park, T., Shuler M., *Biotechnol Progr* 19 (2003): 243–253;
[4] Shim, J., et al., *Curr top in med chem* 3 (2003): 687–703;
[5] Cooper McDonald J., Duffy D.C., Anderson J. R., Chiu D.T., Wu H., Schueller O. J. A., *Electrophoresis* 21 (2000): 27–40;
[6] Giannitsis A. T., *Est Journal of Eng* 17 (2011): 109–139;
[7] Ziółkowska K., Kwapiszewski R., Brzózka Z., *NewJ. Chem*. 35 (2011): 979–990;
[8] D'Orazio P., *Clin Chim Acta* 412 (2011): 1749–1761;
[9] M. L. Chabinyc, D. T. Chiu, J. C. McDonald, A. D. Stroock, J. F. Christian, A. M. Karger and G. M. Whitesides, *Anal. Chem*. 73 (2001): 4491–4498;
[10] K. Liu, Y. Tian, R. Pitchimani, M. Huang, H. Lincoln and D. Pappas, *Talanta* 79 (2009): 333–338;
[11] *Mikrobioanalityka*, red. Brzózka Z., OWPW, Warszawa, 2009;
[12] Chudy M., Grabowska I., Ciosek P., Filipowicz-Szymańska A., Stadnik D., Wyżkiewicz I., Jędrych E., Juchniewicz M., Skolimowski M., Ziółkowska K., Kwapiszewski R., *Anal Bioanal Chem* 395 (2009): 647–668;
[13] Ziolkowska K., Zukowski K., Brzozka Z., Kwapiszewski R., Chudy M., Dybko A., *3-D microsystems made of poly(dimethylsiloxane)*, patent; date of application 18.05.2011;
[14] Ziółkowska K., Stelmachowska A., Kwapiszewski R., Chudy M., Dybko A., Brzózka Z., *Biosens Bioelectron,* (2012), http://dx.doi.org/10.1016/j.bios.2012.06.017;
[15] Ziółkowska K., Chudy M., Dybko A., Brzózka Z., *Challenges of Modern Technology* 2 (2011): 54-61;
[16] L. Y.Wu, D. Di Carlo and L. P. Lee, *Biomed. Microdevices* 10 (2007): 197–202;
[17] E. Cukierman, R. Pankov and K. M. Yamada, *Curr. Opin. Cell Biol.*, 14 (2002): 633–639;
[18] K. M. Yamada and E. Cukierman, *Cell* 130 (2007): 130, 601–610;
[19] L. G. Griffith and M. A. Swartz, *Nat. Rev. Mol. Cell Biol.* 7 (2006): 211–224;
[20] N. Korin, A. Bransky, U. Dinnar and S. Levenberg, *Biomed. Microdevices* 11 (2008): 87–94;
[21] J. Park, F. Berthiaume, M. Toner, M. L. Yarmush and A. W. Tilles, *Biotechnol. Bioeng*. 90 (2005) 632–644;
[22] H. Yu, I. Meyvantsson, I. A. Shkel and D. J. Beebe, *Lab Chip* 5 (2005): 1089–1095;
[23] E. Leclerc, Y. Sakai and T. Fujii, *Biotechnol. Prog*. 20 (2004): 750–755;
[24] Kim L., Toh Y-C., Voldman J., Yu H., *Lab Chip* 7 (2007): 681–694;
[25] B. G. Chung, L. A. Flanagan, S. W. Rhee, P. H. Schwartz, A. P. Lee, E. S. Monuki and N. L. Jeon, *Lab Chip* 5 (2005): 401–406;
[26] C. J. Bettinger, E. J. Weinberg, K. M. Kulig, J. P. Vacanti, Y. D. Wang, J. T. Borenstein and R. Langer, *Adv. Mater*. 18 (2006): 165–169;
[27] W. Gu, X. Zhu, N. Futai, B. S. Cho and S. Takayama, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004): 15861–15866;
[28] R. Z. Lin and H. Y. Chang, *Biotechnol. J*. 3 (2008): 1172–1184;
[29] F. Pampaloni, E. H. Stelzer and A. Masotti, Recent Pat. *Biotechnol.* 3 (2009): 103–117;
[30] A. Y. Hsiao, Y. S. Torisawa, Y. C. Tung, S. Sud, R. S. Taichman, K. J. Pienta and S. Takayama, *Biomaterials* 30 (2009): 3020–3027;
[31] J. B. Kim, *Semin. Cancer Biol*. 15 (2005): 365–377;
[32] J. Debnath and J. S. Brugge, *Nat. Rev. Cancer* 5 (2005): 675–688;
[33] M. D. Tang, A. P. Golden and J. Tien, *J. Am. Chem. Soc*. 125 (2003): 12988–12989;
[34] W. G. Koh and M. V. Pishko, *Anal. Bioanal. Chem*. 385 (2006): 1389–1397;