Validation of a novel perfusion bioreactor system in cancer research

Jasmina Stojkovska[1,2], Jovana Zvicer[1], Milena Milivojevic[3], Isidora Petrovic[3], Milena Stevanovic[3,4,5] and Bojana Obradovic[1]

[1]*Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia*
[2]*Innovation Center of the Faculty of Technology and Metallurgy, Belgrade, Serbia*
[3]*Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia*
[4]*Faculty of Biology, University of Belgrade, Belgrade, Serbia*
[5]*Serbian Academy of Sciences and Arts, Belgrade, Serbia*

Abstract

Development of drugs is a complex, time- and cost-consuming process due to the lack of standardized and reliable characterization techniques and models. Traditionally, drug screening is based on *in vitro* analysis using two-dimensional (2D) cell cultures followed by *in vivo* animal testing. Unfortunately, application of the obtained results to humans in about 90 % of cases fails. Therefore, it is important to develop and improve cell-based systems that can mimic the *in vivo*-like conditions to provide more reliable results. In this paper, we present development and validation of a novel, user-friendly perfusion bioreactor system for single use aimed for cancer research, drug screening, anti-cancer drug response studies, biomaterial characterization, and tissue engineering. Simple design of the perfusion bioreactor provides direct medium flow at physiological velocities (100–250 μm s^{-1}) through samples of different sizes and shapes. Biocompatibility of the bioreactor was confirmed in short term cultivation studies of cervical carcinoma SiHa cells immobilized in alginate microfibers under continuous medium flow. The results have shown preserved cell viability indicating that the perfusion bioreactor in conjunction with alginate hydrogels as cell carriers could be potentially used as a tool for controlled anti-cancer drug screening in a 3D environment.

Keywords: 3D culture; biomimetic bioreactor; tumor model; alginate microfibers

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1. INTRODUCTION

Over the past decade, there has been an increasing need for physiologically relevant 3D cell and tissue culture systems that will provide reliable results and address the *in vitro-in vivo* gap [1,2]. In specific, the traditional approach relying on 2D cell monolayers, in cell-based studies, drug discovery and biomedical research, followed by *in vivo* tests on animals has revealed discrepancy of the results obtained in these two experimental phases. Cells cultured in 2D lose their normal morphology, extracellular matrix (ECM) and cell-to-cell interactions leading to different metabolism and responses as compared to the *in vivo* settings [3,4]. This problem has been particularly recognized in cancer research [2,5], as well as in the fields of nanomaterials and especially in nanotoxicology [1,6]. On the other hand, successful extrapolation of the results obtained in animal studies to humans is less than 8 % [7], due to species-specific differences [8]. More relevant 3D culture systems using human cells and tissues were indicated as a potential solution for these problems [2,5].

Biomimetic bioreactors that imitate environments in native tissues and organs *in vivo* have been developed mainly for tissue engineering purposes [9-11]. Furthermore, these bioreactors can be useful tools also in drug screening, cancer research and characterization of novel biomaterials. As an example, we have previously shown that cytotoxicity studies of Ag/alginate nanocomposite hydrogels in cartilage explant tissue cultures in a biomimetic bioreactor with dynamic

Corresponding author: Jasmina Stojkovska, Innovation Center of the Faculty of Technology and Metallurgy, Belgrade, Serbia; Tel.: +381-11-3303609; Fax: +381-11-3303387
E-mail: jstojkovska@tmf.bg.ac.rs
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compression applied in a regime relevant for articular cartilage have indicated the nanocomposite biocompatibility without signs of cytotoxicity [12]. These results were in accordance with the functionality that these hydrogels exhibited in burn treatments in rats while contrary to moderate cytotoxic effects found in chondrocyte monolayers [12].

In cancer research, multicellular spheroids were recognized as suitable *in vitro* tumor models that mimic some properties of solid tumors such as: (i) spatial organization of cells in layers with different proliferation rates and formation of diffusive gradients of nutrients, oxygen and metabolic waste, (ii) presence of cell-to-cell interactions and secretion of soluble mediators, (iii) expression of specific gene patterns, and (iv) drug resistance [13,14]. However, multicellular spheroid models have several limitations compared to the *in vivo* environment, such as the lack of ECM deposition and spheroid growth limitations over long-term cultivation due to diffusion limitations of nutrients and gases within the spheroids [15]. In order to improve model systems for cancer research, one of the approaches is based on immobilization of cells in biomaterials that mimic ECM, followed by cultivation in biomimetic bioreactors under *in vivo*-like conditions.

Perfusion was found to be one of the key factors for 3D cell and tissue cultivation due to providing efficient mass transport of nutrients and oxygen by convection and controlled levels of hydrodynamic shear stresses exhibited on cells [16]. There are several commercially available perfusion systems such as: 3DCulturePro™ Bioreactor (ElectroForce Systems Group, Eden Prairie, MN), 3D Perfusion Bioreactor (3D Biotek, NJ), U-CUP (CELLEC Biotek AG, Basel, Switzerland), InFlow Perfusion Bioreactor (SKE Research Equipment®, Bollate, Italy), LiveBox1 (IVTech, Massarosa, Italy), and OsteoGen (BiSS TGT, Karnataka, India). Most of these systems are rather complicated for handling especially for users without the technical expertise, such as pharmacists, biologists and medical specialists.

We have recently developed a simple, single-use perfusion chamber as a part of a ready-to-use perfusion system "3D Perfuse". This system is suitable for different sample types and shapes, providing tight sealing and thus medium flow directly through the sample. In this work, we present validation of the novel perfusion system in conjunction with cell immobilization for cancer research. In specific, we have devised a simple immobilization method of cancer cells in alginate microfibers followed by cultivation in the perfusion bioreactor under physiologically relevant medium flowrates.

2. MATERIALS AND METHODS

2. 1. Materials

Low viscosity alginate was purchased from AppliChem (A3249, Darmstadt, Germany). Calcium nitrate tetrahydrate (31218), sodium citrate dihydrate (W302600), trypan blue, MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyl tetrazolium bromide), phosphate buffered saline (PBS), and Trypsin /EDTA were supplied by Sigma (St. Louis, MO). Annexin V (Annexin V Alexa Fluor® 488 conjugate), propidium iodide (PI), high glucose Dulbecco's Modified Eagle's medium with L-glutamine (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin/amphotericin B (100 x antibiotic/antimycotic) were obtained from Invitrogen™ (NY).

2. 2. Cell culture

SiHa cells (ATCC®, HTB-3$5^{TM}$, USA) were cultivated in culture dishes (10 cm in diameter) at 37°C in 10 % CO_2 in the culture medium that is DMEM (4500 mg dm^{-3} glucose, 2 mmol dm^{-3} L-glutamine) supplemented with 10 % FBS and 1× antibiotic/antimycotic. 2D monolayer cultures of SiHa cells served as a controls for all analysis.

When the confluence of about 90 % was reached, the cells were detached by using 0.25 % Trypsin /1mM EDTA. In brief, SiHa cells in culture dishes were washed with 5 ml 1× PBS, treated with 1 ml Trypsin /EDTA for 5 min at 37 °C, after which Trypsin /EDTA was neutralized with DMEM.

2. 3. Production of alginate microfibers with immobilized cells

Before the production of alginate microfibers, SiHa cells from 2D monolayer cultures were detached as described in the Sect. 2.2., followed by cell counting by a Thoma chamber. Na-alginate powder was dissolved in deionized water at a concentration of 2 % w/w and then mixed with a suspension of SiHa cells in the culture medium to obtain final concentrations of 1.5 % w/w alginate and 4×10^6 cells cm^{-3}. Alginate concentration was selected according to our

previous studies of alginate microfiber production by the extrusion technique [17] and immobilization of different cell types in alginate hydrogels [9, 18], while the value for cell density of 1×10^6 cells cm^{-3} was reported for immobilization of NtTera2 cancer stem cells in alginate hydrogels [19]. However, in our preliminary studies the cell density of 4×10^6 cells cm^{-3} was found to be better and therefore was selected for the present study. The cell/alginate suspension was manually extruded through a blunt edge stainless steel needle (25 gauge) immersed in the gelling bath (3 % w/w $Ca(NO_3)_2\times4H_2O$). Due to the exchange of Na^+ with Ca^{2+}, the liquid stream solidified in the gelling bath, thus forming insoluble microfibers with immobilized SiHa cells. The microfibers were left in the bath for 15 min in order to complete gelling and then were washed with the culture medium. The microfibers were then placed into fresh medium and cultivated for the next 24 h. Finally, the obtained microfibers with SiHa cells were divided randomly for: i) cell counting, ii) 2D monolayer cultures, iii) 3D static cultures (microfibers with immobilized cells were cultivated in culture dishes), and iv) cultivation in "3D Perfuse" bioreactors under continuous medium flow. All of the experiments were performed in triplicates.

For cell counting, as well as for 2D monolayer cultures, alginate microfibers were dissolved in 2 % w/v sodium citrate solution (0.1 g of microfibers in 1 cm^3 of the solution) for 10 min at 37°C and then washed two times in the culture medium.

Cell number was estimated by using a Thoma counting chamber and cell viability was assessed by means of trypan blue dye exclusion test. In addition, the rest of the cells retrieved from alginate microfibers was cultivated in culture dishes for 72 h and analyzed for viability.

2. 4. 3D perfusion cultures

Perfusion systems ("3D Perfuse", Innovation Center of the Faculty of Technology and Metallurgy, Belgrade, Serbia) comprising novel single-use chambers [20] were used for cultures of SiHa cells immobilized in alginate microfibers. Each system is provided in a separate pouch, sterile and connected, and comprises one chamber, silicone tubing, two 3-way stopcocks and a medium reservoir. The chamber is configured as two plastic parts connected by silicone tubing that forms a space for sample placement (Fig. 1). Three different tubing diameters can be used (8, 10 and 12 mm inner diameter) while the tubing can be cut to the length appropriate for the particular sample size. In the present experiment, 8 mm inner diameter tubing was used forming a chamber 5 mm in length.

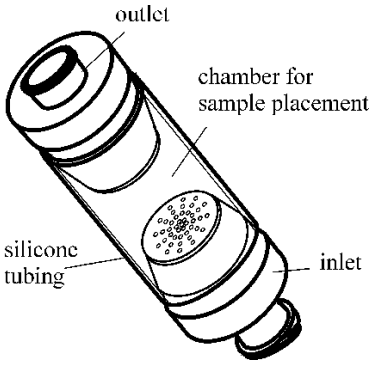

Figure 1. Bioreactor chamber: two plastic halves are fit into a piece of silicone tubing to form a chamber for the sample placement.

2. 4. 1. Setting up the system

A pouch was opened in a laminar hood by using sterile gloves so that the perfusion system can be taken out on a sterile surface. The bioreactor chamber is packed open on the inlet side. Alginate microfibers with immobilized cells (0.5 g) were put into the chamber by forceps and the chamber was closed by forcing the inlet part into the tubing (Fig. 2a-c). A syringe with medium was placed in the inlet stopcock, which was then opened towards the bioreactor chamber and the chamber was filled with medium (direction 1 in Fig. 2d). During that time one port at the medium reservoir was slightly opened to let the air out while filling the system. The inlet stopcock was then opened towards the tubing coil and reservoir, which were then filled with the medium (direction 2, Fig. 2e). Finally, the tubing between the chamber outlet and the medium reservoir was filled by a syringe placed in the outlet stopcock (direction 3, Fig. 2f). The medium volume in the system was about 13 ml.

Three perfusion systems were set up and placed in a humidified 10 % CO_2 incubator at 37°C. Medium perfusion was performed at the flowrate of 0.05 cm^3 min^{-1} by a multichannel peristaltic pump (LabN1, Baoding Shenchen Precision Pump Co., China) placed outside of the incubator (Fig. 2g). Microfibers with immobilized cells under static conditions in 10 cm Petri dishes (0.5 g of microfibers in 13 ml of the culture medium, triplicate) served as a control.

The experiment lasted for 72 h and microfibers retrieved from the perfusion and static cultures were dissolved using Na-citrate as described above. The released cells from microfibers were seeded in culture dishes (35 mm in diameter) at the concentration of 1×10^5 cells cm^{-3} in 3 cm^3 of the culture medium and cultured in a humidified 10 % CO_2 incubator at 37°C for 24 h to allow cell attachment. Also, retrieved cells were used for the apoptosis assay.

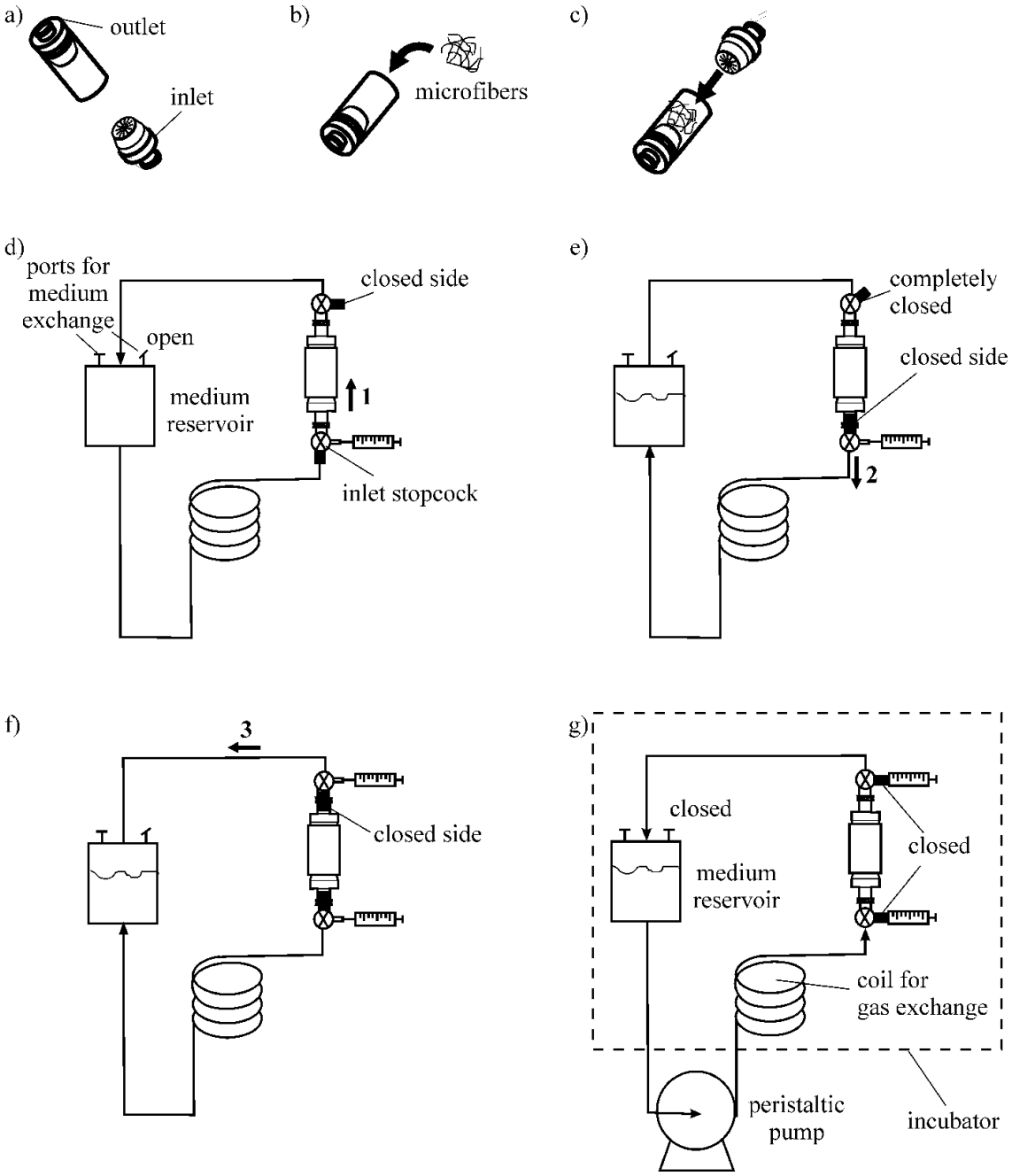

Figure 2. Setting up and filling the system: a) the chamber comes open at the inlet side; b) alginate microfibers are placed in the chamber; c) the chamber is closed by forcing the inlet part into the silicone tubing; d) one of the ports on the medium reservoir is opened, and the chamber is filled with medium in the direction 1 by a syringe placed in the inlet stopcock; e) tubing and the reservoir are filled with medium in the direction 2; f) the outlet tubing is filled with medium in the direction 3; g) the system is placed in a humidified CO_2 incubator and medium flow is provided by a multichannel peristaltic pump.

2. 5 Characterization of cells immobilized in alginate microfibers

2. 5. 1 Optical microscopy

Microfiber diameters were measured by using an optical microscope DM IL LED Inverted Microscope (Leica Microsystems, Germany) at a 10× magnification. The average microfiber diameter was calculated from the measured data of at least 20 microfibers using the Leica Application Suite V4.3.0 software.

2. 5. 2 MTT assay

Viability of the cells within the alginate microfibers was assessed by the MTT assay 96 h after immobilization (24 h in a static culture and 72 h in the perfusion/control static culture). Briefly, the microfibers were incubated for 15 h in the MTT solution (0.5 mg cm^{-3} in the culture medium). Images of formazan crystals produced by metabolically active cells were taken by a DM IL LED Inverted microscope (Leica Microsystems, Germany).

2. 5. 3 Apoptosis assay

Cells were retrieved from 2D cultures by washing with cold PBS, followed by detaching from the plates by using Trypsin /EDTA and then centrifuged for 5 minutes at 1200 rpm followed by resuspension in 1× Annexin binding buffer at the concentration of 1×10^6 cells cm^{-3}. Cells immobilized in alginate microfibers were retrieved by dissolving the alginate hydrogel by the Na-citrate solution as described above followed by resuspension in the buffer. In specific, 5 µl of Annexin V and 5 µl of propidium iodide (PI, 1 mg cm^{-3}) were added to resuspended cells. After incubation for 10 min in the dark at RT, cells were analyzed by a Partec CyFlow® Space flowcytometer (Partec GmbH, Germany). The flowcytometer collected 100,000 events, which were analyzed by using the FloMax software for cytometry (Partec GmbH, Germany).

3. RESULTS AND DISCUSSION

3. 1. 3D cultures

Simple extrusion of the cell/alginate suspension (4×10^6 cells cm^{-3}, 1.5 % w/w alginate) directly into the gelling bath resulted in formation of uniform alginate microfibers (diameter 200 ± 25 µm, Fig. 3a) with immobilized SiHa cells. After 24 h in the culture medium microfibers were dissolved and the retrieved cells were counted. The concentration of immobilized cells was 1.84×10^6 cells cm^{-3} which is significantly lower (~2 fold) than the initial cell concentration in the cell/alginate suspension. These results can be explained by possible cell loss during the extrusion process, as well as negative effects of sodium citrate on cell viability during dissolution of microfibers. However, it should be also noted that some experimental errors are introduced during sampling of wet microfibers, due to the influence of trapped medium between microfibers contributing to the overall hydrogel weight thus decreasing the apparent cell concentration.

Still, the retrieved SiHa cells retained their ability to attach to the cell culture plastic (Fig. 3b) and to proliferate under 2D conditions (Fig. 3c).

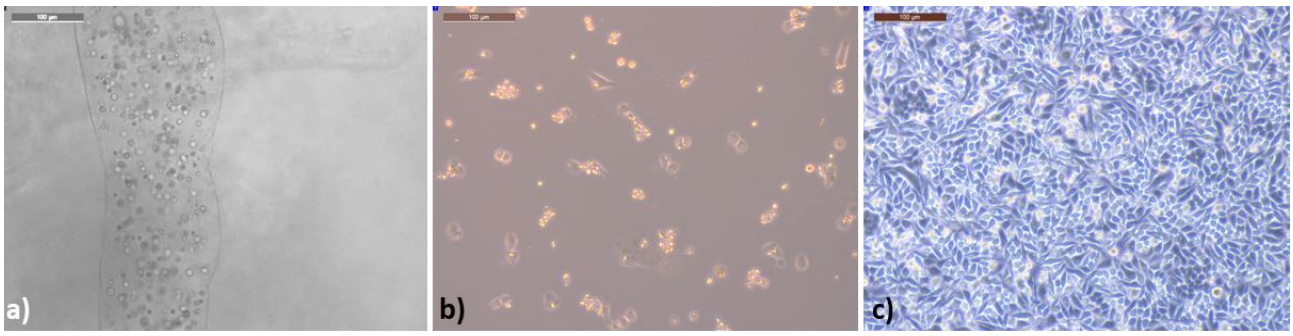

Figure 3. Immobilization of SiHa cells in alginate microfibers: a) optical micrograph of the initial alginate microfiber with immobilized cells; b) SiHa cells retrieved from alginate microfibers after 24 h; c) retrieved SiHa cells after cultivation in a 2D monolayer for additional 72 h (scale bar: 100 µm)

Alginate microfibers with immobilized cells were further cultivated in perfusion bioreactors at the flowrate of 0.05 cm min^{-1}, which corresponded to the superficial velocity of ~15 µm s^{-1}, which is slightly lower than the reported range of blood velocities in capillaries (150 – 250 µm s^{-1}, [21]). Over 72 h of cultivation in the bioreactor as well as under static conditions the microfibers maintained consistency (Fig. 4). In addition, immobilized cells in microfibers in both experimental systems that is under continuous medium perfusion and static conditions appeared to form cell aggregates (Fig 4).

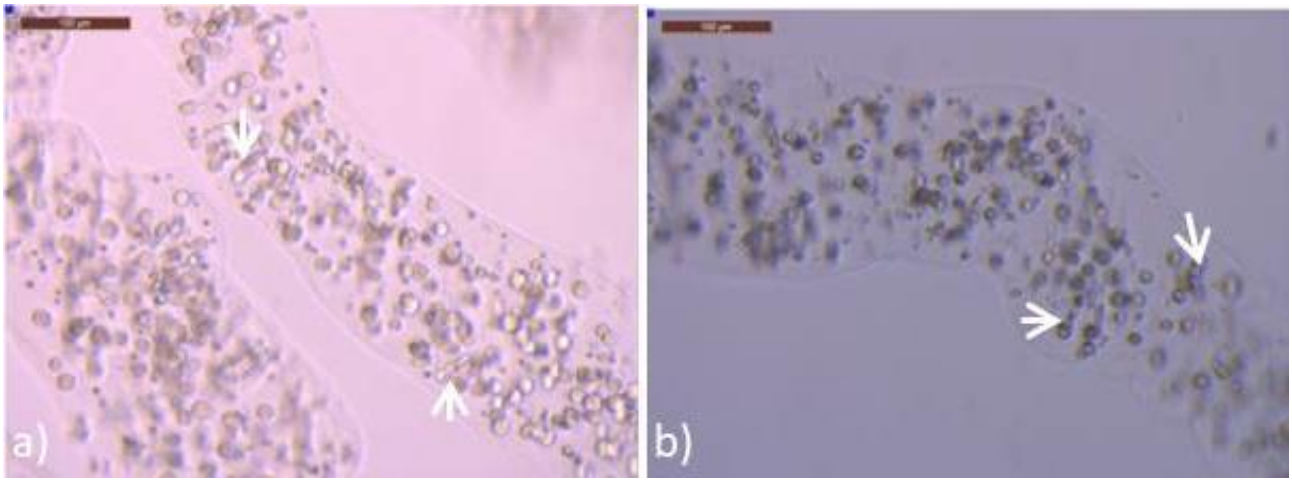

Figure. 4. Alginate microfibers with immobilized SiHa cells cultured for 24 h under static conditions followed by 72 h cultivation under: a) static conditions; b) continuous medium perfusion (scale bar: 100 µm, white arrows designate cell aggregates)

In order to validate viability of immobilized SiHa cells, we have performed the MTT assay directly on microfibers [22]. This assay is based on reduction of the MTT solution to formazan by cells that are metabolically active. Immobilized cells in both experimental systems formed colored formazan crystals within the microfibers (Fig. 5). These results indicate that majority of SiHa cells were viable after 96 h in both 3D cultures, as well as confirmed aggregate formation over time as shown already in optical micrographs (Fig. 4).

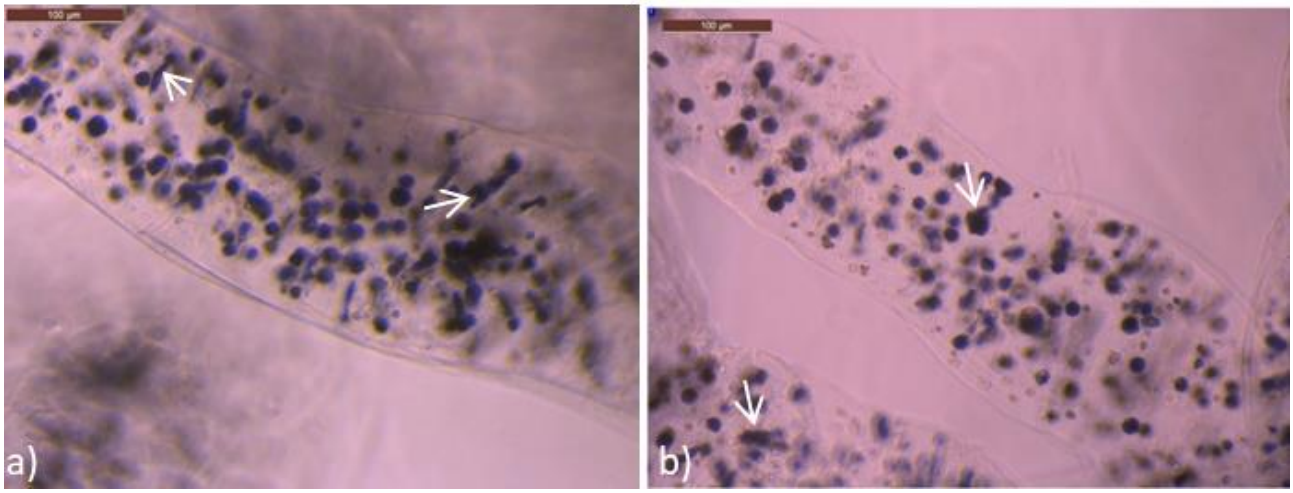

Figure 5. Optical micrographs of MTT-stained viable SiHa cells within alginate microfibers cultured for 24 h under static conditions followed by 72 h cultivation under: a) static conditions; b) continuous medium perfusion; (scale bar: 100 µm, white arrows designate cell aggregates).

Furthermore, the SiHa cells retrieved from alginate microfibers stayed viable and retained their ability to attach to the cell culture plastic (Fig. 6). This result indicates that the presented 3D system supports cancer cell cultivation, which offers possibility to be used for many applications, for example anticancer-drug screening [1,16,23].

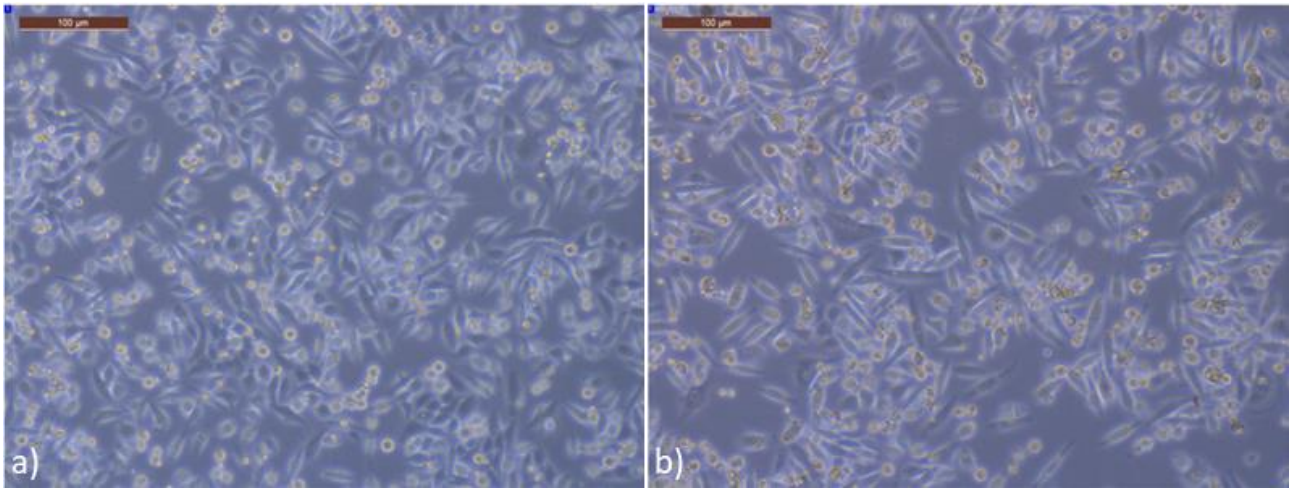

Figure 6. SiHa cells retrieved from alginate microfibers after 3D cultures (96 h in total) and cultured for additional 72 h in 2D monolayer cultures from: a) 3D static culture, b) 3D perfusion culture (scale bar: 100 µm)

3. 2. Apoptosis in SiHa cells in 2D and 3D cultures

In order to elucidate the effect of continuous perfusion on cell death, we have analyzed SiHa cells cultured for 96 h in total in three different settings: i) 2D monolayer cell culture, ii) 3D culture under static conditions and iii) 3D culture for 24 h under static conditions followed by 72 h under continuous perfusion. The cells were collected and subjected to the apoptosis assay (Fig. 7). Majority of cells (99.9 %) cultivated in 2D monolayer cell culture were Anexin V/PI negative, representing the population of living cells.

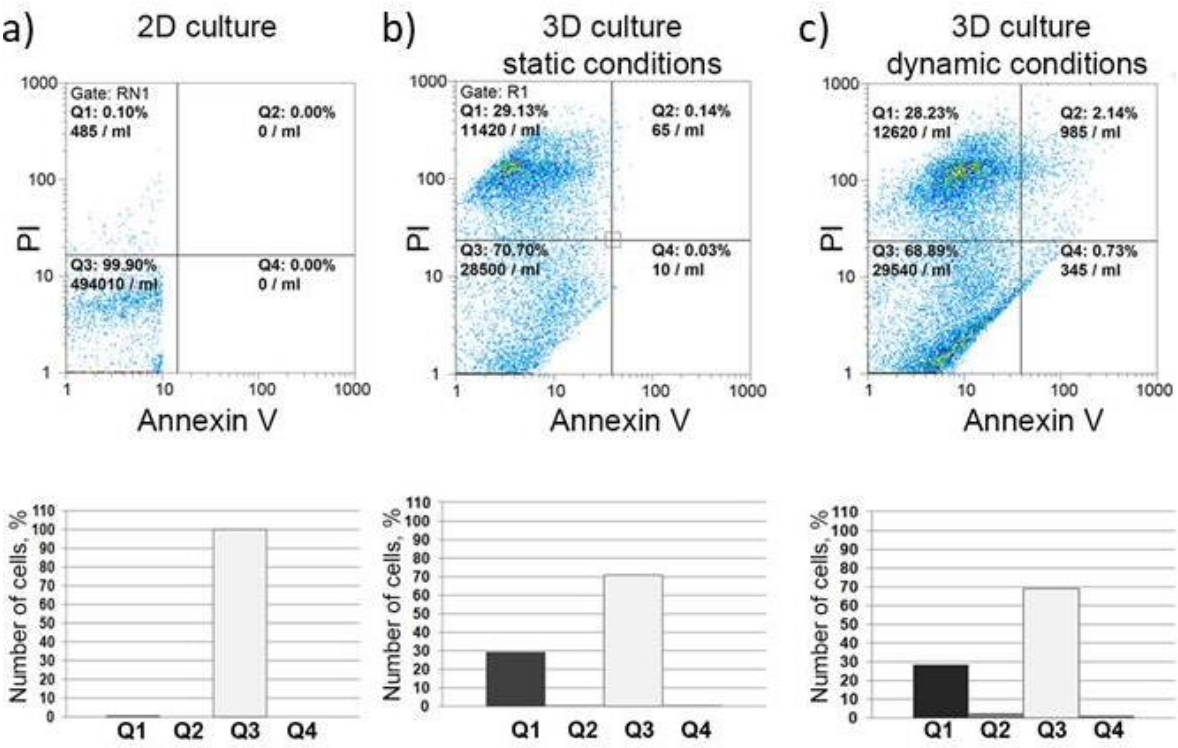

Figure 7. *Apoptosis assay. Carcinoma cell line SiHa was cultivated for 96 h in: a) 2D monolayer culture, b) 3D static culture and c) 3D culture under static conditions for 24 h followed by 72 h under continuous medium perfusion. Flow cytometry analysis of Annexin V-FITC staining and propidium iodide (PI) accumulation was performed. Distribution of cells in each quadrant is presented with representative images and histograms below. Q1: PI+ cells (necrosis); Q2: PI+/Annexin V+ cells (late apoptosis); Q3: PI-/Annexin V- cells (live cells); Q4: Annexin V+ cells (early apoptosis)*

Cultivation under 3D culture conditions, both static and continuous medium perfusion, led to the overall cell death of approximately 30 % (including Anexin V positive, PI positive and double positive cells) without significant differences between these two cultures. Slightly more apoptotic cells were obtained in the perfused culture (~3 % *vs*. 0.2 % in the 3D static cultures), which should be further investigated. The obtained increase in cell death in 3D cultures could be attributed to mass transfer limitations within the alginate hydrogel or by the influence of the alginate microfiber dissolution procedure, which includes cell exposure to the sodium citrate solution. Sodium citrate was shown to induce cytotoxic effects on different cell lines such as a prostate cancer cell line (PC3), gastric cell lines BGC-823 and SGC-7901, and stomach adenocarcinoma cell line (AGS) in dose- and time- dependent manner [24-27]. In addition, effects of sodium citrate on cell viability were shown to depend on the cell growth phase [28]. Our future studies will include assessment of the effects of sodium citrate on SiHa cells and optimization of the alginate hydrogel dissolution procedure or alternatively, hydrogel dissolution by a different agent *i.e*. ethylenediaminetetraacetic acid (EDTA), as proposed in literature [2,29,30]. Finally, possible influences of external (in the medium) and internal (within the alginate hydrogel) mass transfer limitations will be assessed by increasing the medium flowrate and decreasing the microfiber diameter, respectively.

4. CONCLUSION

Biomimetic approaches to cancer research provide possibilities to closely imitate *in vivo* environment in order to obtain more relevant and reliable results. Here, we present a novel perfusion biomimetic bioreactor in conjunction with alginate hydrogels as cancer cell carriers. In this study, SiHa cells immobilized in alginate microfibers stayed viable and metabolically active for 96 h under static conditions as well as exposed to continuous medium flow for 72 h. These results are relevant for potential application of this model system in anti-cancer drug screening. Slightly increased cell death in the 3D system indicated the need for further optimization of operating conditions and/or characterization protocols. Overall, results of this study have shown potentials of the perfusion biomimetic bioreactor for 3D cultures of cancer cells and possibly other tissue models for drug screening and development of personalized therapies.

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SAŽETAK

Validacija novog protočnog bioreaktorskog sistema u ispitivanju tumora

Jasmina J. Stojkovska[1,2], Jovana S. Zvicer[1], Milena Milivojević[3], Isidora Petrovic[3], Milena Stevanović[3,4,5] i Bojana M. Obradović[1]

[1]Tehnološko-metalurški fakultet, Univerzitet u Beogradu, Beograd, Srbija
[2]Inovacioni centar Tehnološko-metalurškog fakultetu, Beograd, Srbija
[3]Institut za molekularnu biologiju u genetičko inženjerstvo, Beograd, Srbija
[4]Biološki fakultet, Belgrade, Serbia
[5]Srpska akademija nauka i umetnosti, Beograd, Srbija

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Razvoj novih lekova je kompleksan, dugotrajan i skup proces usled nedostatka standardizovanih i pouzdanih metoda, i model sistema za evaluaciju lekova.. Tradicionalno, ispitivanje lekova se bazira na *in vitro* analizama korišćenjem dvodimenzionalnih (2D) ćelijskih kultura praćenim *in vivo* testiranjima na životinjama. Nažalost, kada je potrebno primeniti dobijene rezultate na ljude, u oko 90 % slučajeva taj proces je neuspešan. Stoga je veoma važno razvijati sisteme koji će imitirati *in vivo* uslove i na taj način omogućiti dobijanje pouzdanijih rezultata. U ovom radu je razvijen i validiran novi protočni bioreaktor za jednokratnu upotrebu koji je lak za rukovanje i namenjen je za istraživanje malignih ćelija, testiranje lekova, ispitivanje odgovora na anti-tumorske lekove, karakterizaciju novih biomaterijala, kao i za inženjerstvo tkiva. Jednostavan dizajn protočnog bioreaktora omogućava direktan protok medijuma fiziološkim brzinama (100–250 μm s^{-1}) kroz uzorke različitih veličina i oblika. Biokompatibilnost bioreaktora je potvrđena tokom kraktotrajne kulture SiHa ćelija cervikalnog karcinoma imobilisanih u alginatna mikrovlakna pri konstantnom protoku medijuma. Dobijeni rezultati su pokazali da su ćelije ostale vijabilne što ukazuje da protočni bioreaktor zajedno sa alginatnim hidrogelovima kao nosačima ćelija ima potencijal da se koristi za kontrolisano ispitivanje anti-tumorskih lekova u 3D okruženju.

K*ljučne reči*: 3D kultura, biomimični bioreaktori, model tumora, alginatna mikrovlakna