

ENHANCED CHONDROCYTE PROLIFERATION IN A PROTOTYPED CULTURE SYSTEM WITH WAVE-INDUCED AGITATION

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One of the actual challenges in tissue engineering applications is to efficiently produce as high of number of cells as it is only possible, in the shortest time. In static cultures, the production of animal cell biomass in integrated forms (i.e. aggregates, inoculated scaffolds) is limited due to inefficient diffusion of culture medium components observed in such non-mixed culture systems, especially in the case of cell-inoculated fiber-based dense 3D scaffolds, inside which the intensification of mass transfer is particularly important. The applicability of a prototyped, small-scale, continuously wave-induced agitated system for intensification of anchorage-dependent CP5 chondrocytes proliferation outside and inside three-dimensional poly(lactic acid) (PLA) scaffolds has been discussed. Fibrous PLA-based constructs have been inoculated with CP5 cells and then maintained in two independent incubation systems: (i) non-agitated conditions and (ii) culture with wave-induced agitation. Significantly higher values of the volumetric glucose consumption rate have been noted for the system with the wave-induced agitation. The advantage of the presented wave-induced agitation culture system has been confirmed by lower activity of lactate dehydrogenase (LDH) released from the cells in the samples of culture medium harvested from the agitated cultures, in contrast to rather high values of LDH activity measured for static conditions. Results of the proceeded experiments and their analysis clearly exhibited the feasibility of the culture system supported with continuously wave-induced agitation for robust proliferation of the CP5 chondrocytes on PLA-based structures. Aside from the practicability of the prototyped system, we believe that it could also be applied as a standard method offering advantages for all types of the daily routine laboratory-scale animal cell cultures utilizing various fiber-based biomaterials, with the use of only regular laboratory devices.

Keywords: wave-induced agitation, small-scale animal cell culture, CP5 chondrocytes, fibrous-based scaffold, single-use bioreactor

1. INTRODUCTION

Enhancement of adherent cell proliferation inside biomaterial-based three-dimensional structures is one of the current research issues in modern bioengineering focused on development and scale-up of mammalian cell cultures. Typically applied static, i.e. non-agitated, culture conditions generally limit inoculation and proliferation efficiency due to obvious limitation of mass transfer inside biomaterial constructs (Dunn et al., 2006). Each bioprocess development improving cell proliferation in the three-dimensional inner structure of scaffolds is strongly invited to overcome these limitations.

One of the actual challenges in applications of bioengineering focused on tissue engineering is to efficiently produce as many cells as it is only possible, in the shortest time. In the static cultures the production of animal cell biomass in integrated forms (i.e. aggregates, inoculated scaffolds) is limited due to inefficient diffusion of culture medium components observed in such non-mixed culture

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systems, especially in the case of cell-inoculated fiber-based dense 3D scaffolds, inside which the intensification of mass transfer is particularly important (Chung and Burdick, 2008; Marx, 2012). Instead of commonly applied rotating or tumbling stirrers used to induce fluid flow in classical bioreactor systems, continuously oscillating devices can be utilized for gently obtained homogeneous conditions in the systems for *in vitro* culture of fragile animal cells. In such approaches an agitation is achieved by horizontal/vertical oscillations of the culture vessel which is fixed in a rocker unit. The rocking motion is very efficient in generating waves, and the wave-induced motion in the culture container causes agitation of large volumes of culture medium (Fig. 1), and facilitates dispersion of components of a culture microenvironment (i.e. gases, nutrients, extracellularly secreted waste bioproducts) (Eibl et al., 2009).

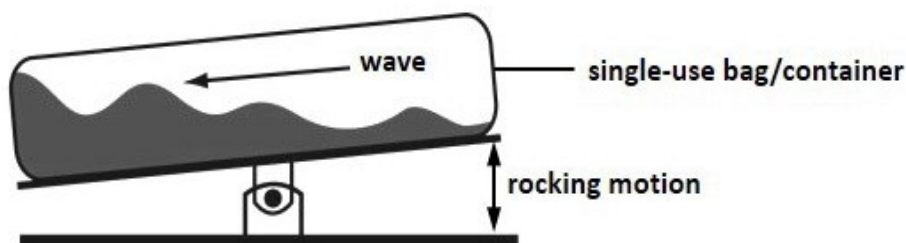


Fig. 1. An idea of a wave-induced agitation by continuous rocking motion of a disposable bag/container (i.e. a single-use bioreactor) fixed in a rocker unit

Presently, such continuously agitated bioreactor systems which utilize effectiveness of waves for culture medium mixing are readily and successfully applied in the wide types of bioprocesses not only focused on animal or insect cells (Eibl and Eibl, 2011) but also cultures of microorganisms (Junge et al., 2013), especially microalgae (Hillig et al., 2014) as well as plant (Georgiev et al., 2008) cells.

The aim of this work was to discuss the applicability of a continuous wave-induced agitation system for intensification of proliferation of chondrocytes (as an example of anchorage-dependent type of animal cells) outside and inside three-dimensional (3D) poly(lactic acid) (PLA) scaffolds. Fibrous PLA-based constructs have been inoculated with CP5 mammalian chondrocytes and then maintained in two independent incubation systems: (i) non-agitated conditions and (ii) culture system with wave-induced agitation. The volumetric glucose consumption rate (r_G) has been analyzed to recognize the metabolic activity of the CP5 cells overgrew the biomaterial structures. The death rate of the CP5 cells has been estimated based on the activity of lactate dehydrogenase (a_{LDH}) released from the cells into culture medium. SEM micrographs of the biomaterials have been used to spot distribution of the cells on the outer surface of the PLA-based constructs maintained in both examined systems.

2. MATERIALS AND METHODS

2.1. CP5 cells and culture medium

The CP5 cell line constituted by the anchorage-dependent, articular cartilage progenitor cells isolated from *Bos taurus* (Holstein-Friesian breed), was used in this work. The CP5 cells have been purchased from European Collection of Authenticated Cell Cultures (ECACC)/Health Protection Agency Culture Collection (Salisbury, UK). The CP5 chondrocytes were maintained in Dulbecco's modified Eagle medium (DMEM; Life Technologies, USA) with high glucose concentration (4.5 g L^{-1}), supplemented with GlutaMAX® (4.0 mM L -glutamine), 10 % fetal calf serum (FCS), $50 \mu\text{L mL}^{-1}$ ascorbate, 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), i.e. zwitterionic buffering agent, and antibiotics ($0.05 \mu\text{L mL}^{-1}$ penicillin, $0.05 \mu\text{L mL}^{-1}$ streptomycin) at $37 \text{ }^\circ\text{C}$, and 5 % of CO_2 according to

procedure applied previously (Pilarek et al., 2014). DMEM, FCS and all used chemicals were obtained from Life Technologies (USA) and were of animal cell culture certified.

2.2. PLA-based fibrous constructs

The PLA-based scaffolds (16 mm diameter, 2 - 3 mm thickness), made of 1.0-2.0 μm thin fibres electrospun in electro-hydro-dynamic atomization (EHDA) process from granulated 20 - 30 kDa PLA (Polysciences, Inc., USA) dissolved in dichloromethane:N,N-dimethylformamide (9:1, v/v) at 7 % according to procedure reported previously by Wojasiński et al. (2014). Prior to using them in culture systems, PLA scaffolds were washed and sterilized by immersion in 70 % ethanol and next dried in aseptic air for 1 h at room temperature.

2.3. Prototyped wave-induced agitation system

A prototyped wave-induced agitation system has been presented in Fig. 2. The thermostatic chamber, made with stainless-steel double walls, with inner sizes suitable for standardized multiwell-plates or flasks typically used for small-scale animal cell cultures, has been fixed on a rocking unit (Rocking Platform Shaker KL-942; JW Electronic, Poland). The constant temperature (37.0 ± 0.05 °C) inside the chamber has been obtained according to continuously flow of heated water from thermostat (Immersion Thermostat E5; Medingen, Germany) through inner space between two walls of the chamber. The rocking unit provided the possibility of smooth rocking-angle regulation from $-1^\circ/1^\circ$ to $-10^\circ/10^\circ$ with rocking-frequency range of 2 - 60 cpm.

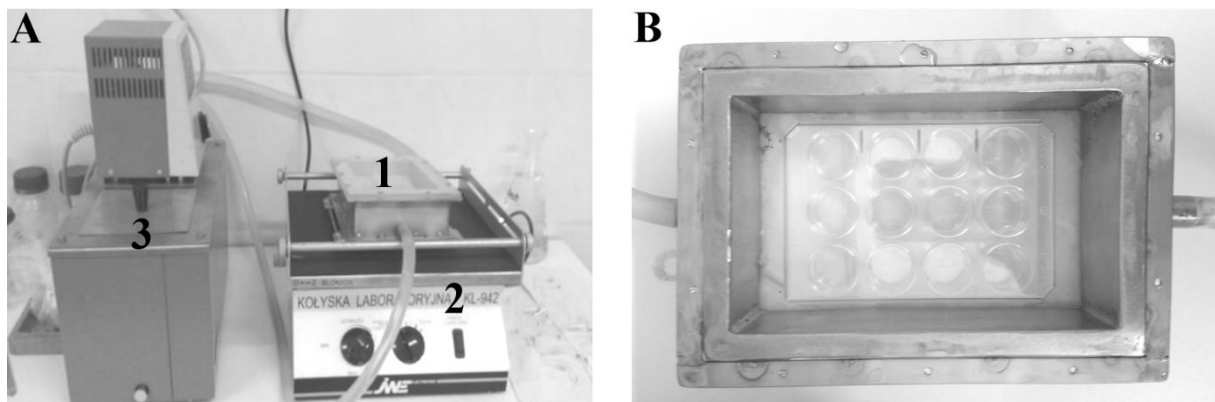


Fig. 2. The prototyped wave-induced agitation system for milliliter-scale cultures of CP5 chondrocytes on PLA-based scaffolds: A - the experimental setup including the thermostatic chamber for incubation of standardized culture multiwell-plates/flask (1), the rocking unit providing the wave-induced agitation (2) and the water thermostat (3); B - the standardized culture multiwell-plate fixed inside the double walls thermostatic chamber

2.4. Experimental procedure

The CP5 cells were cultured on PLA-based fibrous scaffolds immersed in 1.0 mL of DMEM in 24-deepwell-plates (Becton-Dickinson, USA) used as milliliter-scale culture vessels. Two independent culture systems have been maintained: (i) non-agitated conditions and (ii) culture with wave-induced agitation (rocking angle: $-5^\circ / 5^\circ$, rocking-frequency: 20 cpm). Both of compared culture systems were triplicated to obtain statistically credible values of quantitative parameters characterizing the proliferation of cells. Inoculum of CP5 cell line was prepared from standard nearly, i.e. 75 - 80 %, confluence cultures, passaged every 4 - 5 days. Briefly, cells were washed with PBS (Life

Technologies, USA), incubated in 0.05 % trypsin (Life Technologies, USA) for ca. 3 min at 37 °C, the number of cells was estimated using Malassez hemocytometer (Brand, Germany), then cells were suspended in DMEM and finally pipetted into the 24-deepwell-plates with scaffolds to obtain initial density of $5.0 \cdot 10^5$ cells mL^{-1} . The samples of culture medium have been harvested every 24 hours from both of studied culture systems containing proliferating CP5 cells for determination of the glucose concentration and the lactate dehydrogenase activity. Then additionally, to prevent cultured CP5 cells from detrimental effects caused by low concentrations of nutrients and/or high levels of extracellular secreted toxic metabolites, or cell-lysis products soluble in culture medium, the DMEM-based medium was fully exchanged for the fresh culture medium after every 24 hours of the experiment in all inoculated wells of the multiwell-plates.

2.5. Analytical procedures

Glucose concentration has been determined according to the enzymatic BioMaxima glucose-determination kit (BM-G; BioMaxima, Poland) procedure. The analytical method which has been applied in the studies is based on the results of two subsequently proceeded enzymatic reactions catalyzed by glucose oxidase (first step) and peroxidase (second step of the procedure). In the first step of the reaction, glucose is oxidizing into gluconic acid with release of hydrogen peroxide. In the second step, hydrogen peroxide molecules are selectively reacting with phenol and 4-aminoantypirine which finally results in colored chinonoimine appearance in the samples. The absorbance of chinonoimine has been measured at 500 nm vs. reference absorbance of pure BioMaxima reagent sample (i.e. without chinonoimine). Glucose concentration in the samples of culture medium (C_G) was calculated based on known values of absorbance measured for: the sample of culture medium (A_{CM}), as well as the standard ($1.0 \text{ mg} \cdot \text{mL}^{-1}$) solution of glucose (A_G), with the following equation:

$$C_G = \frac{A_{CM}}{A_G} [\text{mg} \cdot \text{mL}^{-1}] \quad (1)$$

Based on the results of the C_G , the volumetric glucose consumption rate (r_G) can be easily determined as follows:

$$r_G = \frac{C_G}{t} \left[\frac{\text{mg} \cdot \text{mL}^{-1}}{\text{day}} \right] \quad (2)$$

where t represents culture period (i.e. day).

Activity of lactate dehydrogenase (a_{LDH}) has been determined according to the BioMaxima lactate dehydrogenase activity determination kit (BM-LDH; BioMaxima, Poland) procedure. The analytical method is based on the results of the reduction of pyruvic acid catalyzed by lactate dehydrogenase (LDH) released from damaged cells, in the presence of NADH. LDH released from cells reduces pyruvic acid. The changes of absorbance in time (i.e. 1 min) measured at 340 nm are related to a_{LDH} in a sample of the culture medium as following:

$$a_{LDH} = \frac{V_T \cdot 10^5}{\epsilon_{NADH}^{340nm} V_S} \cdot \frac{\Delta A}{t} = 267.2 \frac{\Delta A}{t} [\mu\text{kat} \cdot \text{L}^{-1}] \quad (3)$$

where:

V_T - total volume of reaction mixture ($V_T = 1.01 \text{ mL}$), ϵ_{NADH}^{340nm} - molar absorbance coefficient for NADH at 340 nm ($\epsilon_{NADH}^{340nm} = 6.3 \cdot 10^2 \text{ m}^2 \text{ mol}^{-1}$), l - light path length (i.e. thickness of the measuring cuvette; $l = 1 \text{ cm}$), V_S - volume of sample ($V_S = 0.01 \text{ mL}$), ΔA - change of absorbance, t - time ($t = 60 \text{ s}$).

2.6. SEM-imaging procedure

The fibrous PLA-based scaffolds were analyzed with the Phenom (FEI, USA) scanning electron microscope (SEM) supported with producer's image software. The samples selected for SEM analysis firstly were incubated in 0.5 % OsO₄ for 1 h at 4°C, then dehydrated/desiccated with anhydrous ethanol, next automatically dried (Leica EM CPD300, Germany), and finally coated with 15-nm layer of gold (K550 Emitech, USA).

3. RESULTS

To show the feasibility of the wave-inducing agitation for metabolic activity of CP5 cells overgrowing the PLA-scaffolds, we have analyzed r_G and a_{LDH} in samples of the culture medium maintained in both compared, i.e. non-agitated and wave-agitated culture systems. r_G may be recognized as the parameter which quantitatively characterizes metabolic activity of the maintained cells. LDH is an enzyme found in all kinds of animal cells. Because it is released during cell or tissue damage, it may be recognized as a marker of cell destruction or damage. Due to this, a_{LDH} , i.e. the activity of intracellular LDH released from cells into the culture medium, may be identified as quantitative parameter for identification of the culture system which negatively or detrimentally influences cells which are incubated in defined culture system, with an effect of extracellularly leaking of such enzyme-based indicator.

The values of r_G determined for both compared culture systems have been presented in Fig. 3. Significantly higher values of r_G have been observed for CP5 cells maintained within PLA-scaffolds in the culture system with wave-induced agitation when compared to rather moderate values of r_G values noted for the same cells cultured in static, i.e. non-agitated, conditions. In the case of 4 - 5 days of culture the values of r_G measured for the system with wave mixing (i.e. 1.8, 2.1 and 2.2 mg·mL⁻¹·day⁻¹ in consecutive days of culture) were up to 3× higher than the r_G values noted for the static system (i.e. 0.67, 0.71 and 0.83 mg·mL⁻¹·day⁻¹ in consecutive days of culture).

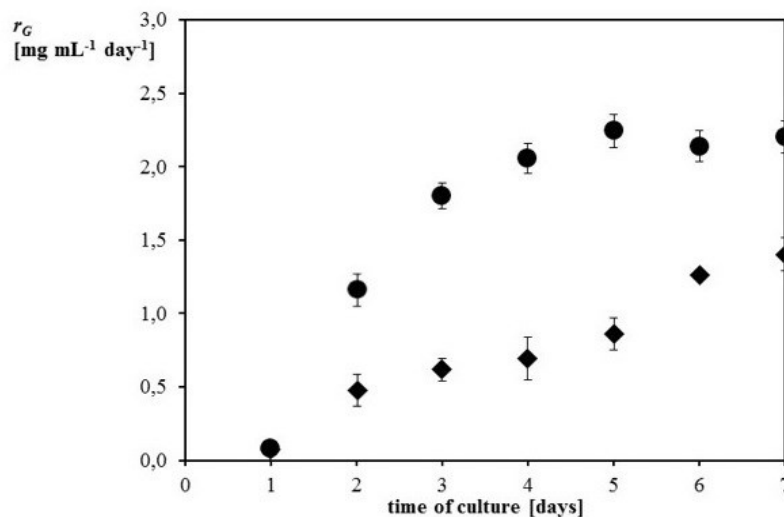


Fig. 3. Values of r_G determined for CP5 cells cultures within PLA-based scaffolds in two compared systems: non-agitated culture (◆) and culture with wave-induced agitation (●)

The values of a_{LDH} determined in culture medium from two compared culture systems have been presented in Fig. 4. The level of a_{LDH} determined in samples from the wave-agitated culture system was significantly lower for the whole time-range of compared cultures. In the case of 4 - 6 days of culture the values of a_{LDH} measured for the static culture system (i.e. 6.8, 6.9 and 7.3 μ kat·L⁻¹ in consecutive

days of culture) were at least twice higher than the a_{LDH} values noted for the wave-mixed system (i.e. 3.2, 3.4 and 3.4 $\mu\text{kat}\cdot\text{L}^{-1}$ in consecutive days of culture).

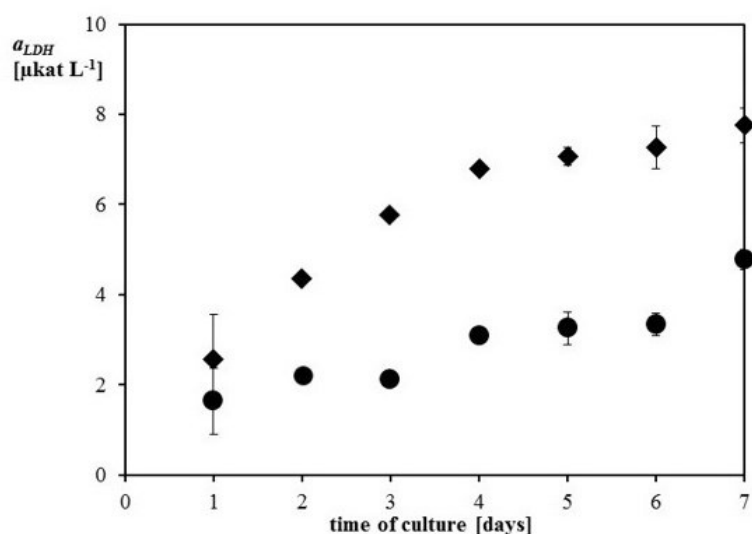


Fig. 4. Values of a_{LDH} determined in samples of culture medium harvested in parallel from two compared systems: non-agitated culture (◆) and culture with wave-induced agitation (●), which have been used for CP5 cells maintaining within PLA-based scaffolds

The SEM micrographs of the biomaterials have been used to spot distribution of the cells on the outer surface of the PLA-based constructs maintained in both examined systems. In the case of culture conditions characterizing our experiments, CP5 chondrocytes adhered to fibres of PLA on the outer surface of used biomaterial constructs after 7 days of cultures in both compared culture systems have been presented in Fig. 5. It can be clearly seen that CP5 cells have proliferated more intensively on the outer surface of the biomaterial maintained in the culture system with wave-induced agitation (Fig. 5B) when compared to rather limited distribution of the cells in the case of constructs incubated in the non-agitated culture system (Fig. 5A). Robust and intensive proliferation of CP5 chondrocytes on the PLA-based biomaterial observed in the wave-agitated system resulted in progressive invasion of the outer area, i.e. as overgrowth of the constructs by the cells and formation of the large cell-clusters on the surface of the fibrous biomaterial.

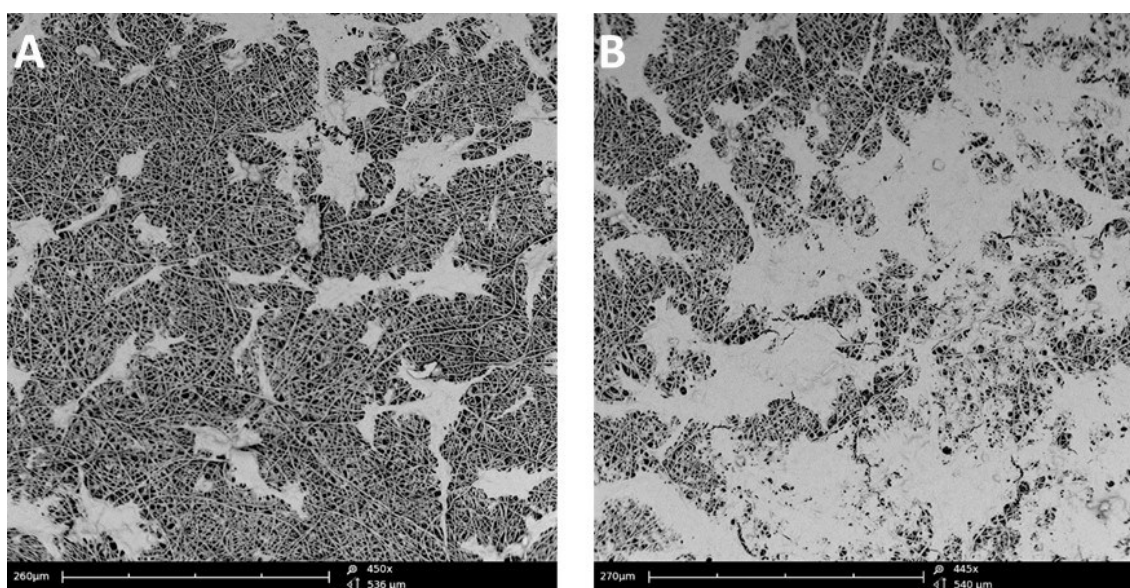


Fig. 5. SEM micrographs of the PLA-based scaffolds inoculated with CP5 chondrocytes and maintained in two compared systems (7th day of cultures): non-agitated culture (A) and culture with wave-induced agitation (B)

4. DISCUSSION

Due to fragility of animal cells resulting from lack of cell walls, which triggers extreme sensitivity of animal cells to shear stress forces generated in typical (i.e. mechanically agitated) cultures, the culture systems providing low-grade forces are needed as proper environment for animal cells *in vitro* propagation. The wave-induced culture system should be taken into consideration as the suitable solution for cells cultured within biomaterial-based constructs, in which gradients of ingredients of culture medium, extracellularly secreted waste metabolites as well as O₂ and CO₂, are usually observed inside the biomaterial and in its vicinity. Mass transfer limitations are identified as substantial problems in static (i.e. non-agitated) conditions typically applied in small-scale (i.e. mililiter-scale) cultures of adherent mammalian cells (Marx, 2012; Matsuura, 2006). Bearing all this in mind, we propose the prototyped continuously agitated mililiter-scale system which utilizes effectiveness of waves for culture medium mixing as the system which can intensify the yield of various types of bioprocesses proceeded with animal cells, especially those which are prepared with application of biomaterial-based 3D scaffolds.

Chondrocytes, as the CP5 cells, have been chosen to perform experiments because they are usually *in vitro* cultured within 3D scaffolds in high density of extracellular matrix. A 3D environment of the culture system is necessary for the maintenance of the native phenotype by isolated chondrocytes cultured *in vitro*, as chondrocytes maintained in typical monolayered form tend to dedifferentiate into fibroblast form (Noriega et al., 2013). Previously, the 3D scaffolds made of micrometre-scale diameters PLA fibres produced by electrospinning process were successfully investigated (e.g. Kwon et al., 2013; Li et al., 2006; Pilarek et al., 2014; Wojasiński et al., 2014) as a synthetic polymeric biomaterial for chondrocyte implant development, whose properties, such as mechanical strength, degradation rate and dimensions can all be easily controlled. There is a distinct need to develop alternative techniques of mass transfer intensification within such 3D dense biomaterial-based constructs/implants to prevent the expansion of detrimental effects caused by progressive necrosis of the cells (Keeney et al., 2011; Pilarek et al., 2014).

High rates of r_G , which occurred in the case of CP5 chondrocytes cultured in the prototyped system with wave-induced agitation, during almost the whole time span (i.e. from 2nd day until end) of the experiment (see Fig. 3) may be the result of high metabolic activity of the cells in culture conditions provided by the wave-induced agitation in contrast to rather moderate levels of r_G values noted for the same cells but maintained in the reference non-agitated conditions. The exception to this are similar values of r_G estimated for both compared systems on 1st day of cultures, which probably resulted from the effects of the adaptation of cells to the culture conditions after passaging during lag-phase.

The determination of intracellular LDH activity, i.e. a_{LDH} , in a culture medium may be recognized as the quantitative method of the death rate of CP5 cell determination because LDH is only leaked from damaged cells (Chan et al., 2013). Significantly lower values of a_{LDH} determined in the samples of culture medium harvested from the system with wave-induced agitation, compared to up to three-times higher values of a_{LDH} in the samples of medium from non-agitated system (see Fig. 4) prove the lack of negative or detrimental influences of culture conditions on maintained cells provided by the wave-induced agitation applied in our prototyped system. We are also hypothesizing that the wave-induced agitation results in intensification of the flow of culture medium through spaces between fibres inside the 3D structure of PLA-based constructs used as scaffolds for proliferating CP5 cells. Therefore more homogenous culture conditions have been probably obtained in the studied culture system if related to the static, i.e. non-mixed, conditions applied in the reference culture system. Accordingly, the mass transfer limitations inside the used biomaterial constructs, e.g. inefficient diffusion of nutrients to cells, have been overcome in the presented culture system mixed by the wave-induced agitation.

As was presented in the results section, the growth of CP5 cells maintained in non-mixed conditions has been rather limited (see Fig. 5A). Robust and intensive proliferation of CP5 chondrocytes, as well as enhanced cell-cluster formation on the fibrous PLA-based scaffolds, have been achieved in the wave-induced culture system (see Fig. 5B). The phenotype of the CP5 chondrocytes on the PLA scaffolds shown by SEM is rather not typical for the native morphology in cartilage extracellular matrix. But the morphology of cells cultured under experimental conditions is in accordance with the results of previously published studies focused on proliferation of cartilage cells on fibrous scaffolds (Hogrebe et al., 2017; McCullen et al., 2012; Pilarek et al., 2014). Such phenotype differences were probably caused by two factors. One of them is the progenitor-nature of the cells applied as inoculum (Dowthwaite et al., 2004). The second reason can be interpreted as the mechanical influence of culture conditions on proliferating biomass in the presented *in vitro* system with gentle wave-type mixing. The flow of culture medium forced by continuous wave-induced agitation intensifies the mass transfer inside 3D constructs but also can additionally influence chondrocyte morphology according to possible mechanical stimulation of proliferating cells. Such hypothetical effects are consistent with conclusions considered previously by other authors (e.g. Chung and Burdick, 2008; Noriega et al., 2013; Vunjak-Novakovic et al., 1999), that *in vitro* cultured isolated chondrocytes should be maintained under culture conditions supporting mechanical stimulation of the cells because mechanical stimuli (e.g. hydrodynamic effects caused by mixing of culture medium) have been recognized as one of the key factors which stabilizes morphology of the cells and influences positively the mechanical properties of cartilage implants. Summarizing, the results of enhanced CP5 cell proliferation outside and inside 3D fibrous-based PLA-constructs when incubated in the proposed prototyped simple small-scale system with the wave-type mixing are proving hypothetic applicability of such a culture system for a range of animal/human cells (e.g. fibroblasts, osteoblasts, vascular cells, etc.) which are *in vitro* cultured within various porous polymeric bioresorbable scaffolds utilized in tissue engineering.

5. CONCLUSIONS AND OUTLOOK

Summarizing, the following conclusions can be drawn based on the results of the experiments presented and discussed above:

- significantly higher values of r_G , denoting high metabolic activity of cells, have been determined for CP5 chondrocytes cultured in the prototyped system with wave-induced agitation than for the same cells cultured in the reference non-mixed conditions;
- up to three-times lower values of a_{LDH} (i.e. intracellular enzyme marker) have been determined in the samples of culture medium harvested from the system with wave-induced agitation, compared to values of the parameter obtained for the samples of medium from non-agitated system;
- higher metabolic activity (represented by values of r_G) and lower level of activity of intracellular enzymatic marker (i.e. a_{LDH}) determined in samples of culture medium harvested from the cultures of CP5 chondrocytes proceeded in the system with wave-induced agitation unambiguously indicate that such a system can be recognized as the suitable environment for 3D cultures of CP5 chondrocytes;
- maintaining of CP5 chondrocyte-seeded fibrous PLA-based 3D scaffolds under conditions provided by the low-shear culture system with wave-induced agitation results in enhanced proliferation of cells on the surface of the biomaterial constructs.

Aside from the practicability of the prototyped system, further studies on quantitative characteristic of mass transfer in the presented prototyped milliliter-scale culture system with wave-induced agitation must be performed to fully recognize and identify in detail the advantages of the system. Nevertheless we believe that the developed culture system could also be applied as a standard method offering

advantages for all types of the daily routine laboratory-scale animal cell cultures utilizing various fibre-based biomaterials, with the use of only regular, and typically available in laboratory, devices.

This work has been supported by the budget sources for The National Centre for Science, Poland, Grant no. DEC-2015/17/B/ST8/00631.

SYMBOLS

A_{CM}	absorbance of culture medium
A_G	absorbance of standard solution of glucose
C_G	glucose concentration in the samples of culture medium, $\text{mg}\cdot\text{mL}^{-1}$
l	light path length, cm
r_G	volumetric glucose consumption rate, $\text{mg}\cdot\text{mL}^{-1}\cdot\text{day}^{-1}$
t	time, s
V_S	volume of sample, mL
V_T	total volume of reaction mixture, mL

Greek symbols

a_{LDH}	activity of lactate dehydrogenase, $\mu\text{kat}\cdot\text{L}^{-1}$
ΔA	change of absorbance
$\epsilon_{NADH}^{340\text{nm}}$	molar absorbance coefficient for NADH at 340 nm, $\text{m}^2\cdot\text{mol}^{-1}$

Superscripts

340nm	wavelength of peak absorption of NADH
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Subscripts

CM	culture medium
G	glucose
LDH	lactate dehydrogenase
$NADH$	reduced form of nicotinamide adenine dinucleotide
S	sample
T	total (e.g. total volume)

Abbreviations

$3D$	three-dimensional
$BM-G$	BioMaxima glucose concentration determination kit
$BM-LDH$	BioMaxima lactate dehydrogenase activity determination kit
$CP5$	mammalian (<i>Bos taurus</i>) chondrocyte progenitor cell line
$DMEM$	Dulbecco's modified Eagle medium
$EHDA$	electro-hydro-dynamic atomization
FCS	fetal calf serum
$HEPES$	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (zwitterionic-based buffering agent)
LDH	lactate dehydrogenase
$NADH$	reduced form of nicotinamide adenine dinucleotide
PBS	phosphate-buffered saline buffer
PLA	poly(lactic)acid
SEM	scanning electron microscopy

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Received 06 October 2016

Received in revised form 08 May 2017

Accepted 24 May 2017