POLY(L-LACTIDE-CO-GLYCOLIDE) MICROPARTICLES EMULSIFIED BY MIXING AND IN A MICROFLUIDIC DEVICE FOR POTENTIAL BOTTOM-UP BONE TISSUE ENGINEERING

Stanisław Marecik* [iD], Małgorzata Krok-Borkowicz [iD], Elżbieta Pamuła [iD]

AGH University of Krakow,
Faculty of Materials Science and Ceramics,
Department of Biomaterials and Composites,
al. A. Mickiewicza 30, 30-059 Krakow, Poland
*E-mail: smarecik@agh.edu.pl

Abstract

The aim of this study was to obtain degradable poly(L-lactide-co-glycolide) (PLGA) microparticles (MPs) with a controlled size for bottom-up bone tissue engineering. The particles were produced using the classical single water/oil emulsification method by mixing with a magnetic stirrer and by using a novel approach based on the application of a microfluidic device. This study involved a thorough investigation of different concentrations of PLGA and poly(vinyl alcohol) (PVA) during microparticle fabrication. The oil phase was PLGA dissolved in dichloromethane or ethyl acetate at 1%, 2% and 4% w/v concentrations. The water phase was an aqueous solution of PVA at concentrations of 0.5%, 1%, 2%, 2.5%, 4% and 5% w/v. The size and size distribution of the MPs were evaluated with an optical microscope. Obtained MPs were incubated in contact with osteoblast-like MG-63 cells and after days 1 and 3, the cell viability was evaluated using the reduction of resazurin and the fluorescence live/dead staining. The results showed that for each concentration of PVA, the size of the MPs increased with an increase in the concentration of PLGA in the oil phase. The MPs obtained with the use of the microfluidic device were characterized by a smaller size and lower polydispersity compared to those obtained with emulsification by mixing. Both methods resulted in the generation of MPs cytocompatible with MG-63 cells, what paves the way to consider them as scaffolds for bottom-up tissue engineering.

Keywords: microparticles, poly(L-lactide-co-glycolide) (PLGA), water/oil emulsification, microfluidic device, bottom-up tissue engineering

Introduction

Bone tissue engineering is promising in the treatment of bone tissue defects resulting from trauma, infections, or tumour resection [1]. Tissue engineering is based on two approaches, the first being the traditional top-down approach, using well-defined porous scaffolds supplemented with growth factors on which cells are deposited, cultured, and finally expected to form bone tissue. All these phenomena are correlated with the degradation of the scaffold material [2]. However, this classical approach has some limitations, such as difficulties in cell penetration to the central part of the scaffold, poor nutrients diffusion, and lack of proper vascularization [3]. A different strategy that solves these problems is the bottom-up approach, which is based on the creation of larger tissue structures by the assembly of microscaffold-cell constructs [4]. These microscaffolds can have a form of degradable microparticles on which cells can be deposited and cultured in static or dynamic conditions, thus assuring better diffusion of nutrients and waste removal. Furthermore, these microparticles can be loaded with drugs with a defined release time or with biologically active particles such as hydroxyapatite to ensure better osseointegration [5].

The material that demonstrates very good performance in bone regeneration is poly(L-lactide-co-glycolide) (PLGA), which is a biocompatible and biodegradable linear polyester [6]. It is possible to control its degradation time through the lactide to glycolide ratio in the polymer composition [7]. PLGA has many advantages, such as easy control of size, shape, and physical properties, which make it attractive for use in bone tissue engineering.

The results obtained in our group on PLGA microparticles show that they support adhesion, growth, and osteogenic differentiation of mesenchymal stem cells and can form microparticle/cell/extracellular matrix constructs suitable for bottom-up tissue engineering [8].

PLGA microparticles can be obtained by various techniques. One of them is a batch water/oil (W/O) emulsification method by mixing [9]. This is a method that allows to produce microparticles, but with high polydispersity. To achieve better homogeneity of the resulting samples, a microfluidic device can be used to produce microparticles [10]. Microfluidic devices operate on the basis of a co-flow of two immiscible phases [11] and produce microparticles by squeezing of the dispersed phase by the water phase. We hypothesize that the use of microfluidic device, due to better control of flow parameters, is expected to enhance the uniformity of PLGA microparticles, providing a platform that holds promise for improved cytocompatibility in bottom-up bone tissue engineering.

The aim of the study was: (1) to optimize the parameters of PLGA MPs manufacturing by a single water/oil emulsification method by mixing to produce microparticles with the lowest polydispersity, that can be achieved with this approach; (2) to use these conditions in the microfluidic device to generate MPs presumably with even lower polydispersity; and (3) to test the cytocompatibility of the obtained microparticles with MG-63 osteoblast-like cells.

Materials and Methods

Preparation of PLGA microparticles

The microparticles (MPs) were obtained by two techniques: single emulsification by mixing with a magnetic stirrer and a microfluidic device (RayDrop, Fluigent). In the case of emulsification by mixing (FIG. 1), to prepare the oil phase, PLGA (85:15, M_n = 100, M_w/M_n = 2.0, Center of Polymer and Carbon Materials of the Polish Academy of Sciences, Zabrze, Poland) was dissolved in dichloromethane (DCM, POCH Basic) at concentrations of 1%, 2% and 4% w/v. As the water phase, 0.5%, 1%, 2%, 2.5% and 4% w/v aqueous solutions of poly(vinyl alcohol) (PVA, Mowiol 4-88, Sigma-Aldrich) were prepared. PLGA solutions were added to a water phase while mixing at a speed of 800 rpm on a magnetic stirrer (MS-52M, Jeio Tech) at room temperature. The emulsions were then stirred for 24 h to evaporate the solvent. After this time, the MPs were drained under vacuum and dried in an incubator at 37°C for 24 h.

In the second production technique, using a microfluid device (RayDrop, Fluigent, FIG. 2A); the oil phase was PLGA dissolved in ethyl acetate (EA, POCH Basic) at a concentration of 2% w/v. As a water phase, an aqueous solution of PVA at a concentration of 2% w/v was prepared. First, the flow rate in the jetting regime (FIG. 2B) was determined by setting the flow rate to 100 µl/min and for the dispersed phase to 20 µl/min. Then the flow velocities of both phases were simultaneously reduced to 10 µl/min for the continuous phase and 0.6 µl/min for the dispersed phase. As a result, the flow in the dripping regime (FIG. 2C) was obtained, thus producing microparticles. The microparticles then fell into a 2% w/v PVA solution in water, which was stirred at a speed of 800 rpm on a magnetic stirrer. After the process was completed, the particles were stirred for another 24 h to evaporate the EA. Then they were drained under vacuum and dried in an incubator for 24 h at 37°C.

FIG. 1. Scheme of single emulsification method by mixing with a magnetic stirrer.
EA – ethyl acetate
PLGA – poly(L-lactide-co-glycolide)
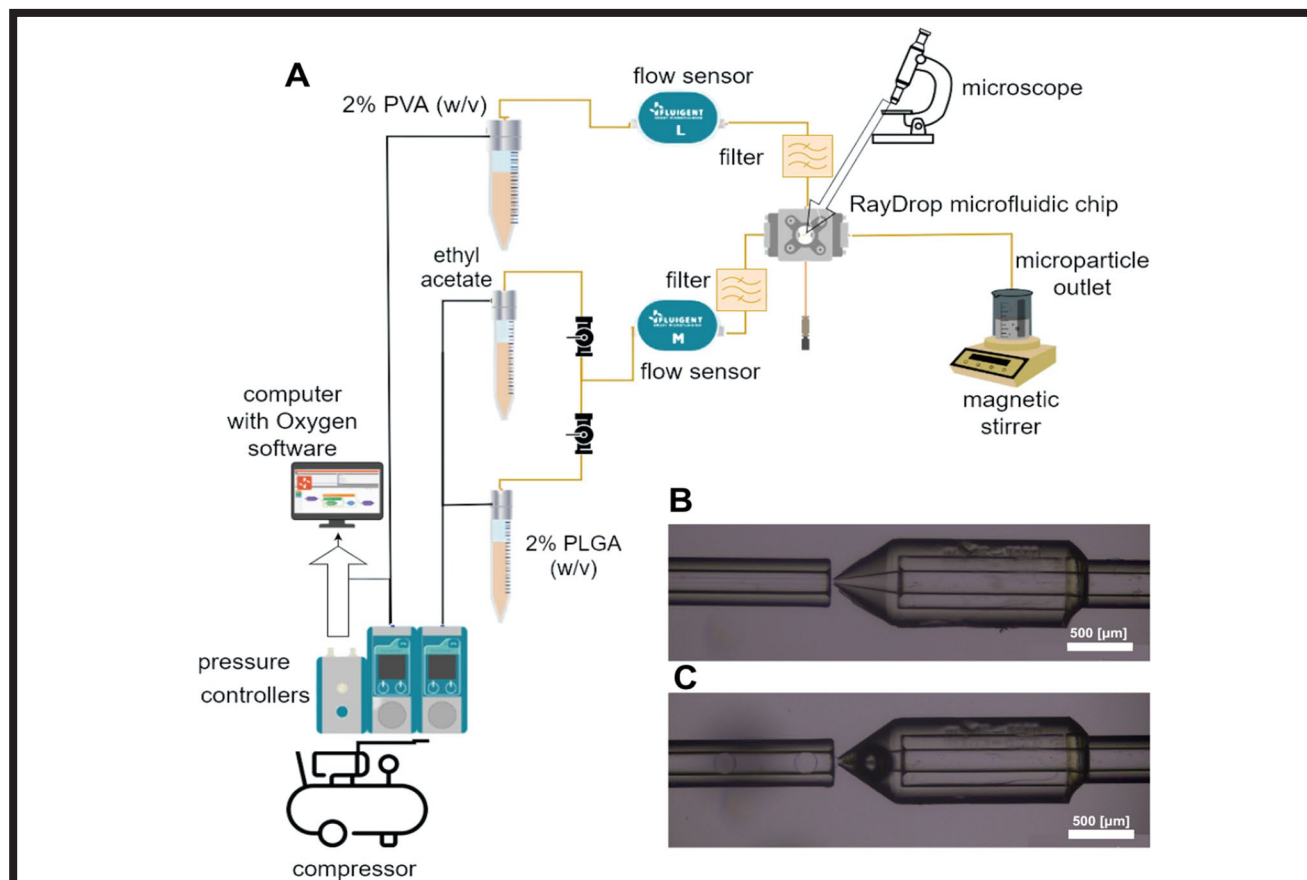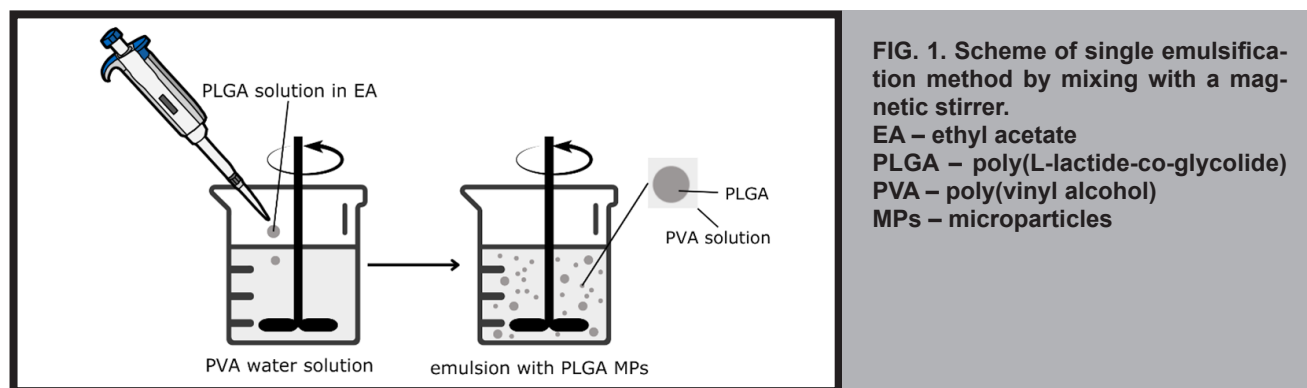PVA – poly(vinyl alcohol)
MPs – microparticles

FIG. 2. Scheme of a microfluidic device RayDrop with accessories (adapted from [12]) (A); phase flow regime inside the RayDrop chip chamber during manufacturing of PLGA microparticles in jetting regime (B) and in dripping regime (C).

Microscopic observations

The MPs morphology was assessed with an optical microscope (Axiovert, Zeiss, magnification: 75X), and ImageJ software was used to measure the size of microparticles (n = 50).

In vitro studies

The MPs were sterilized by immersion in 70% ethanol for 24 h and irradiated with a UV lamp for 30 min. MG-63 osteoblast-like cells (ATCC® CCL-1™, American Type Culture Collection) were cultured in minimal essential medium (MEM, PAN BIOTECH) in contact with MPs on a 24-well plate (Avantor, VWR) at 37°C and 5% CO_2. For each of the well, 2 mg MPs and 500 μl of cell suspension in MEM (16 000 cells/ml) were added and cells were cultured for 1 and 3 days. During incubation, the culture plates were placed on a shaker (PS-3D Sunflower Mini-Shaker, Grant Instruments), with a speed of 30 rpm, to ensure dynamic conditions.

Cell viability was tested with resazurin reduction and live-dead fluorescence staining. The metabolic activity Alamar-Blue test (resazurin-based, Sigma-Aldrich) was carried out using 5% resazurin solution; 300 μl of the solution was added to each of the wells, then incubated for 2 h at 37°C, 5% CO_2. The solution was then transferred to a black 96-well plate (Nunc), and fluorescence was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm (FluoStar Omega, BMG Labtech). The percentage of resazurin reduction was calculated using the formula (1):

$$\%Rezasurin\ Reduction = 100\% \cdot \frac{F_s - F_c}{F_{100\%} - F_c} \qquad (1)$$

where F_s – fluorescence of the sample, F_c – fluorescence of 5% of AlamarBlue solution in a cell-free medium, $F_{100\%}$ – fluorescence of 5% of AlamarBlue solution in a medium after complete reduction at 120°C for 15 min.

In order to assess the arrangement of cells around the MPs and to distinguish between live and dead cells, live/dead staining was performed. This test consisted of adding 300 μl of 0.1% propidium iodide (Sigma Aldrich) and 0.1% calcein AM (Sigma Aldrich) solution in PBS to the wells, then incubated in the dark for 10 min. Subsequently, cells were observed under a fluorescent microscope (Axiovert 40 CFL, Zeiss).

Statistical analysis

The obtained data were checked for normal distribution, the Shapiro-Wilk and Lilliefors test were used. In addition, quantile-quantile plots were prepared. Then, the mean, the standard deviation (SD), and the coefficient of variation (CV) were calculated. The CV is a statistical measure that allows to assess the variability of data in relation to the mean. It is particularly useful when comparing data sets with different means. It was calculated from the formula (2):

$$CV = \frac{SD}{\bar{X}} \cdot 100\% \qquad (2)$$

where: CV – coefficient of variation, SD – standard deviation and $\bar{X}$ – mean.

All calculations and statistical analysis were performed using OriginPro 2023b software.

Results and Discussion

Properties of microparticles

The results show that for each of the PVA concentrations in the water phase, as the PLGA concentration increased, the size of the MPs also increased. As detailed in TABLE 1 and FIG. 3, the highest MPs diameters, equal to 133.5 ± 27.2 μm, were found for microparticles produced when PVA concentration in the water phase was 5% and PLGA concentration in the oil phase was 4%. The smallest MPs, equal to 35.9 ± 9.2 μm, were formed when they were produced with the use of 0.5% PVA in the water phase and 1% PLGA in the oil phase.

TABLE 1. Summary of PLGA MPs size obtained by emulsification by mixing; SD – standard deviation, CV – coefficient of variation.

PVA concentration [%]	PLGA concentration [%]	Mean [μm]	SD [μm]	CV [%]
0.5	1	35.9	9.2	31
	2	56.7	10.8	26
	4	126.0	22.2	25
1	1	48.9	17.3	39
	2	69.7	11.1	24
	4	99.2	30.7	35
2	1	42.6	9.2	28
	2	64.5	12.4	26
	4	90.8	19.4	28
2.5	1	74.6	15.3	27
	2	79.6	25.2	36
	4	98.3	16.3	25
4	1	70.4	23.6	37
	2	87.7	20.2	29
	4	121.7	22.5	26
5	1	60.6	11.9	27
	2	77.9	25.6	37
	4	133.5	27.2	27
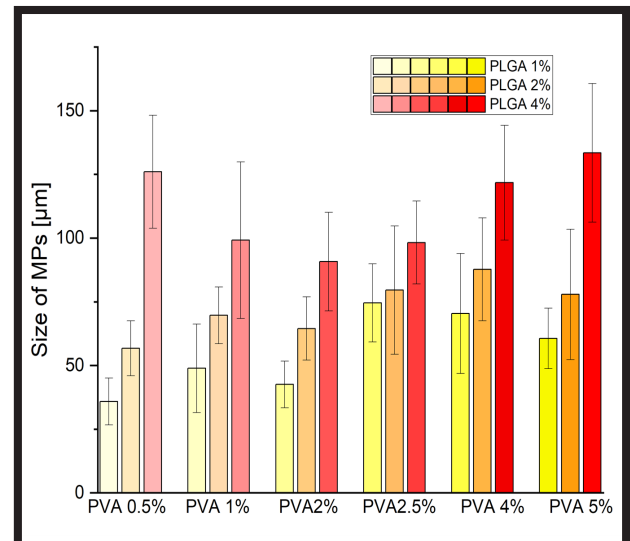
FIG. 3. Sizes of MPs obtained by single emulsification by mixing: the concentration of PLGA in the oil phase was 1%, 2% and 4%, while the concentration of PVA in the water phase was 0.5%, 1%, 2%, 2.5%, 4% and 5%.

The coefficient of variation in MPs diameters was between 24% and 39%, which shows that using the emulsification method by mixing, microparticles with a large but reproducible dispersion can be obtained.

Using the microfluidic device, the sample was obtained using a 2% PVA solution in the water phase and a 2% PLGA solution in the oil phase. The mean diameter of MPs was equal to 43.2 ± 3.2 µm, and the coefficient of variation was 7%, which was the lowest result of all microparticles obtained during the study. The MPs produced in the microfluidic device were characterized by a smaller size and a lower polydispersity as compared to those produced by batch emulsification.

The size of the MPs obtained with the microfluidic device was 43.2 ± 3.2 µm, which was similar to the MPs with 42.6 ± 9.2 µm size obtained with 1% PLGA concentration in the oil phase and 2% PVA concentration in the water phase. Therefore, these two sample types were selected for *in vitro* studies, and their size with standard deviation is presented in FIG. 4A.
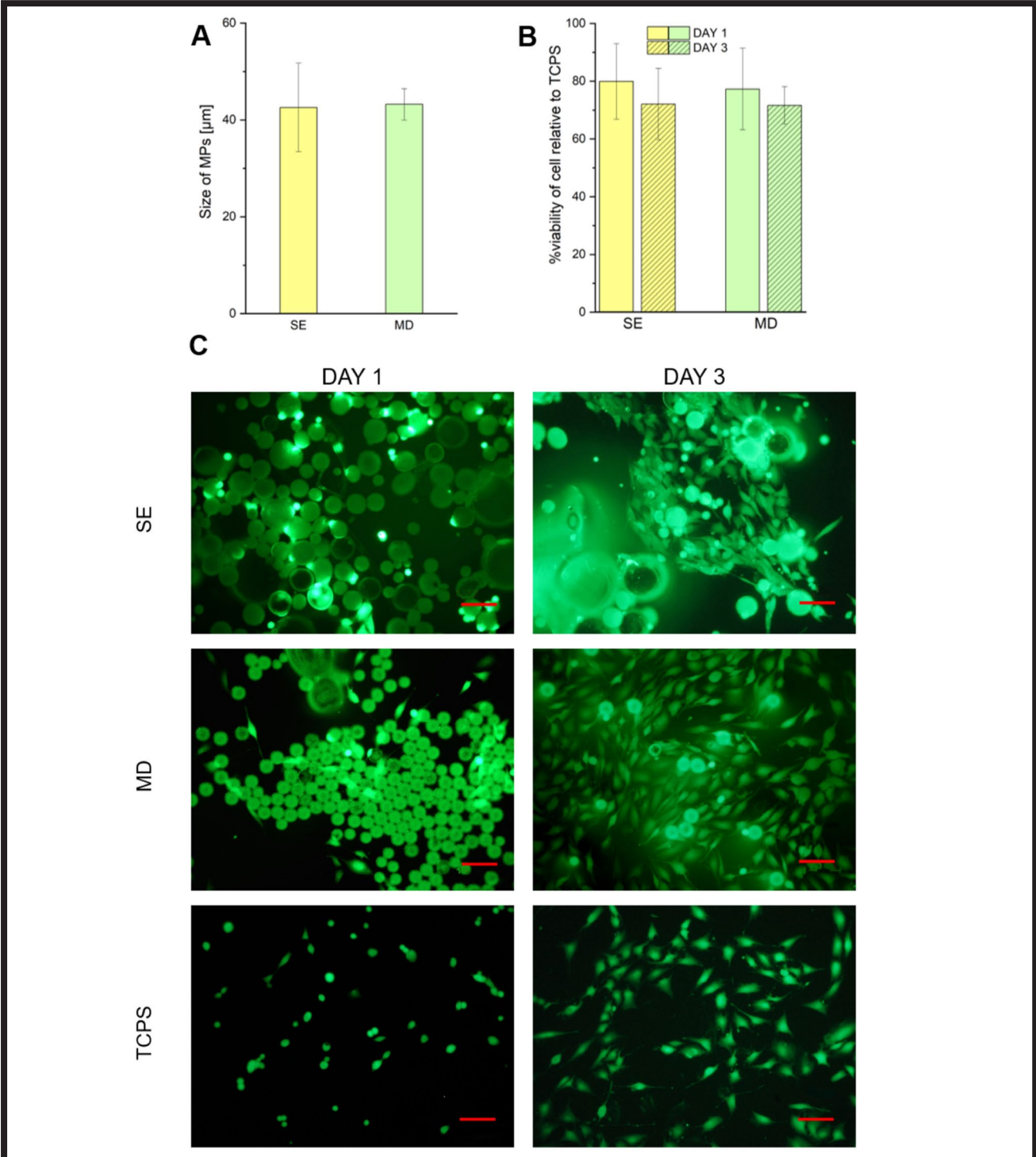

FIG. 4. Size of MPs used in in vitro studies (A); AlamarBlue test results, cell viability relative to control TCPS samples (B); Live/dead staining results after day 1 and day 3: SE – single emulsification, MD – microfluidic device, TCPS – tissue culture polystyrene control sample, scale bar = 100 µm (C).

In vitro studies

To assess the suitability of the materials in the context of bone tissue engineering, *in vitro* studies were conducted. AlamarBlue test showed that for the MPs samples studied in contact with MG-63 cells, cell growth was in the range of 70-80% of control conditions (cells cultured on tissue culture polystyrene, TCPS) (FIG. 4B). Interestingly, in our experiment carried out for the TCPS control sample, the measured resazurin reduction ranged from 2.30% to 2.56%. As shown in our previous studies for a similar number of seeded MG-63 cells, but in static conditions, the resazurin reduction on day 1 was approximately 15-25% and on day 3 it was approximately 30% [13-15]. Lower proliferation in the experiment reported here may be the result of the dynamic culture conditions, because the culture plates were placed on the shaker, which was expected to provoke cell aggregation around microparticles. Nevertheless, it has been shown that MPs produced by emulsification and using a microfluidic device had a similar effect on MG-63 cell viability and are cytocompatible, according to ISO 10993-5 [16].

The viability of cells was confirmed by live/dead staining (FIG. 4C). It was found that after day 1 and 3, the cells grew normally for the control sample and they were stained green, thus alive. For both types of MPs, cells were clustered around the MPs. However, in the areas where the MPs were too dense and the cells did not have space to grow, no cells were observed. Interestingly, no dead red-stained cells were seen, which means that the MPs were not cytotoxic.

Conclusions

PLGA microparticles were obtained with emulsification by mixing and by using a microfluidic device. The microparticles were characterized taking into account their diameter and coefficient of variation. It was shown that the size of microparticles can be controlled by the concentration of PLGA in the oil phase: the higher the concentration, the larger the size. Interestingly, increased concentration of PVA in the water phase has a minor effect on particle size. The MPs obtained by mixing are more polydispersed (coefficient of variation between 24% and 39%), in contrast to those produced using a microfluidic device, which had a coefficient of only 7%. It means that the microparticles produced using the microfluidic device are more homogenous in size. *In vitro* studies of the obtained materials were carried out under dynamic conditions, which significantly reduced MG-63 cells proliferation, but allowed the cellular aggregates to be obtained around the MPs. Viability tests showed that cells seeded on larger particles proliferate better than those seeded on smaller particles. PLGA MPs were found to be cytocompatible with osteoblast-like cells, as shown by the resazurin reduction test and live/dead staining.

Acknowledgements

ORCID iD

S. Marecik: https://orcid.org/0009-0009-7729-424X
M. Krok-Borkowicz: https://orcid.org/0000-0002-9415-0282
E. Pamuła: https://orcid.org/0000-0002-0464-6189

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