OPTIMIZING MANUFACTURING CONDITIONS OF POLYMER MICROSPHERES AS CELL CARRIERS FOR MODULAR TISSUE ENGINEERING

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Abstract

Microspheres (MS) made of biostable polymer, namely polystyrene, have been used as substrates for cell culture enabling rapid cell expansion in dynamic conditions. However, due to non-resorbability, polystyrene (PS) MS when repopulated with cells cannot be directly used in tissue engineering. Our concept was to produce MS from resorbable polymer – poly(L-lactide-co-glycolide) (PLGA) as a support for adherent cells, e.g. osteoblasts. We hypothesize that such MS can be applied to the injured site to act as cell carriers or as modules for modular tissue engineering (MTE). In this article, we present the results of optimizing the PLGA MS manufacturing conditions via oil-in-water emulsification. Due to such a technique, MS with the required size, size distribution and properties suitable for cell culturing can be obtained. Three parameters of the oil-in-water emulsification were examined: the stirring speed of a water phase during MS manufacturing, the surfactant concentration, i.e. poly(vinyl alcohol) (PVA) in a water phase and concentration of PLGA in dichloromethane (DCM) as an oil phase. The results proved that the 7.5% PLGA concentration in DCM solution as an oil phase, the 0.5-2% concentration of PVA solution as a water phase and the stirring speed of water phase of 1000 rpm provided MS with the 160 μm mean diameter, which is suitable for cell culture. Moreover, the developed sieving and cleaning procedures were efficient to collect MS with the mean diameter of 280 μm, the more coherent size distribution and the ability to sink in the cell culture medium. The presence on the bottom of cell culture wells is crucial for MTE.

Keywords: *modular tissue engineering, microspheres, cell culture, oil-in-water emulsification, poly(L-lactide-co-glycolide) (PLGA)*

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Introduction

Apart from classical tissue engineering (TE) which uses macroscopic porous scaffolds seeded with cells, a new approach called modular tissue engineering (MTE) has recently been proposed [1]. The main idea of MTE is to mimic natural tissues and use microscale parts (named also microtissues) that can be assembled into bigger and more complex, dense and organomimetic structures [2–5]. These structures may form cell aggregates, cell sheets, or cells seeded on microcarriers and microspheres (MS). MS can be made of natural and synthetic materials (glass, polymer, ceramics) [6]. The most common MS that are used for the cell expansion in dynamic conditions are made of polystyrene (PS) [7]. With regard to MTE, the main advantage of PS MS is the high surface area accessible for cells in a comparatively low volume [8] while the disadvantage is non-resorbability.

Poly(L-lactide-*co*-glycolide) (PLGA) is widely used as a raw material for medical devices production as it has appropriate mechanical properties and is biocompatible with tissues in different applications. PLGA degrades by hydrolysis of ester bonds into oligomers and, finally, to monomer acids: lactic and glycolic which enter the Krebs cycle and are removed from the body as CO_2 and H_2O [9–10].

PLGA MS can be produced by various methods, such as: emulsification, spray-drying, phase separation, hot-melt extrusion, inkjet printing, gelation, grinding, coacervation, electrospray, supercritical fluid mixing, microfabrication [6].

The oil-in-water emulsification can be used to obtain PLGA MS on a laboratory scale and with multiple parameters controlled. It was reported that the increase in polymer molecular weight and its concentration in the oil phase raised the average MS diameter [11]. Increasing the oil phase volume in the water phase may slightly enlarge the MS diameter but their shape is less regular [12]. Also selecting a proper solvent is highly important the lower the boiling point of the organic solvent, the faster the MS formation [6]. The water phase also gives possibilities to control the emulsification process. For successful MS formation a surfactant addition is required. Poly(vinyl alcohol) (PVA) is commonly used and its concentration in the water phase may also affect the MS diameter – the higher PVA concentration in the water phase, the lower diameter and the narrower diameter distribution [6]. Additionally, the water phase volume increase can lower the MS diameter, however for very high values it causes the MS deformation [12].

The aim of our experiments was to optimize the PLGA MS manufacturing process so as to obtain two batches of particles with the diameters of ca. 150 μm and ca. 250 μm, respectively. In order to obtain MS with the desired mean diameter and the diameter distribution, three parameters were examined: the stirring speed of the water phase, the surfactant concentration, i.e. PVA in the water phase, and the PLGA concentration in the oil phase. The other parameters, such as: the oil and water phases volumes, the beaker and propeller geometry, the PLGA and PVA molecular weight, the lactide to glycolide ratio in PLGA, and the solvent type were constant and are not reported in this article, having been optimized during the preliminary studies. Dichloromethane (DCM) was chosen as a solvent due to its high volatility facilitating the faster MS formation. Additionally, the cleaning process was developed to dispose of the MS with the microstructure and properties inapplicable to cell cultures, e.g. MS with the high diameter dispersion or floating on the aqueous medium surface.

Materials and Methods

Materials

PLGA (85:15, M_n = 100 kDa, M_w = 210 kDa) was synthesized at the Center of Polymer and Carbon Materials, Polish Academy of Sciences, Zabrze, Poland and kindly provided by Prof. P. Dobrzyński. DCM (Avantor Performance Materials) and PVA (Mowiol® 4-88, M_W ca. 31 kDa, Sigma Aldrich) were used.

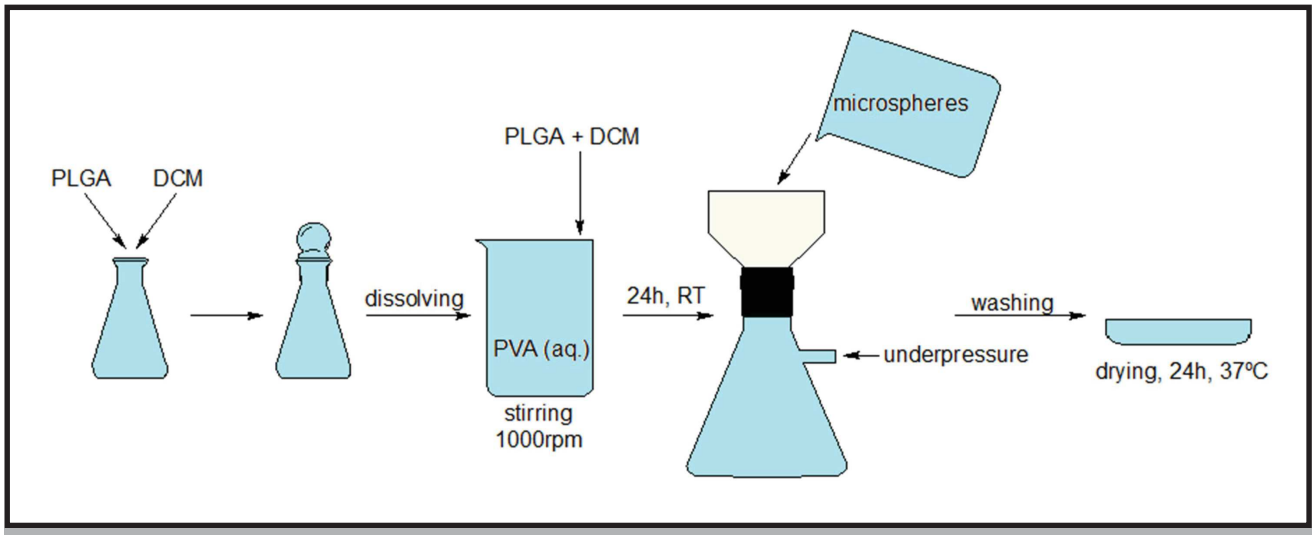

FIG. 1. Scheme of PLGA MS manufacturing by oil-in-water emulsification.

Methods

MS manufacturing by oil-in-water method

To obtain MS, the oil-in-water emulsification was used (FIG. 1). The required amount of PLGA was weighed on an electronic laboratory balance. Under a fume hood, a propeller was put in a glass bottle, then DCM was poured. A ground glass stopper was put to prevent the DCM evaporation. The bottle was placed on a magnetic stirrer and it was turned on to make the propeller rotate. Subsequently, PLGA was added. Such a sequence prevented the polymer particles sticking to the propeller or the glass bottle and assured the PLGA proper dissolution. The glass stopper was inserted into the bottle neck promptly and protected with Parafilm to diminish the DCM evaporation, which would adversely change the ultimate PLGA concentration. The applied oil phase was the PLGA in DCM solution at the 5-10% concentration range.

The water phase was prepared by dissolving PVA in water to obtain the 4% solution. Similarly, the beaker with the propeller was prepared, PVA weighed, a small amount of water added and the stirring started. PVA was added, then the bottle was slowly filled with water to avoid sticking PVA to the bottle walls or the propeller. The bottle was left overnight and then the obtained solution was filtered with a filter paper to remove trace amounts of undissolved PVA. The obtained solution was stored at 4°C in the capped bottle additionally protected with Parafilm and then used as a concentrated stock solution, due to its longer time of preparation as compared to the oil phase. Prior to every synthesis, an exact amount of the PVA solution was diluted to the desired concentration (0.1-4%). The 50 ml water phase was poured into the 100 ml beaker. The process was performed at room temperature.

For the MS synthesis, the beaker containing the water phase was placed on the magnetic stirrer (JeioTech, Model MS-52M) and the propeller was added. While adding the oil phase (always 1 ml with an automatic pipette and the same type of tip) near the beaker wall, the water phase was stirred (at a defined speed of 100-2000 rpm). Placing the oil phase precisely on the beaker wall or in the beaker center resulted in a very low MS production efficiency and a high number of inhomogeneous particles or fibrous structures. The MS were left overnight to solidify via the DCM evaporation. Subsequently, they were vacuum filtered and washed with distilled water multiple times to ensure the PVA residues removal. Then the MS were moved to a Petri dish and left to dry at 37°C for 24 h.

MS sieving and cleaning

In order to obtain MS free of defects such as fibers or foils and endowed with the defined and coherent diameters, the additional process of sieving and cleaning was required. Furthermore, some MS revealed air bubbles entrapped inside, which made them float on the water surface or the cell culture medium. This behavior is highly disadvantageous regarding the culture conditions of bone cells which need a support to adhere and grow. That is why the dried MS were sieved using a grid of 200 µm to collect the MS of higher diameters. Subsequently, the MS fraction was added to a beaker with distilled water, stirred manually, and left for 5 min. After that, the MS which sank to the beaker bottom were collected with a 10 ml pipette. Finally, the MS were dried and stored at 4°C for further studies.

Determining the MS microstructure and size

The MS were observed with an optical digital microscope Keyence VHX-900F. For each sample batch, the diameter of 600 individual MS was measured with the device software and Fiji (ImageJ) software. The data was analyzed by the Shapiro-Wilk test (Origin Pro 2020 9.7.0.188 Academic) to check normality. Since the distribution failed the Shapiro-Wilk test, the results were displayed as histograms and the median was calculated.

Results and Discussion

To obtain MS with the desired average diameter and diameter distribution suitable for bone MTE, three parameters were examined: the stirring speed of the water phase during MS manufacturing, the surfactant concentration, i.e. PVA in the water phase, and the PLGA concentration in the oil phase.

Stirring speed

During the oil-in-water emulsification, the stirring speed has a significant impact on the shear of oil phase flux and on the MS diameter. In this experiment, the PLGA/DCM solution ratio was set at 7.5% and the PVA concentration at 1%, while five different stirring speeds of the water phase were tested: 100 rpm, 250 rpm, 500 rpm, 1000 rpm and 2000 rpm. It was found that the100 rpm stirring speed was too low to form MS – only fibers and irregular foils were obtained. The results showed that all the MS were transparent and exhibited a smooth appearance (FIG. 2, left column).

The results presented in histograms (FIG. 2, right column) showed that for the steering speed of 250 and 500 rpm the MS sizes were similar but the size distribution changed. The percentage of MS with the diameter over 325 µm dropped from 71.5 to 50.6 and the contribution of MS with lower diameters increased. For the steering speed of 1000 rpm a much higher differences were observed. The shape of histogram changed, and the percentage of 115 and 175 µm MS equaled 39%. The highest stirring speed of 2000 rpm resulted in even a higher percentage of MS with the low diameter. The MS of 175 µm or below in diameter represented 72.2% of all the formed MS. Thus, for further studies, the steering speed of 1000 rpm was selected.

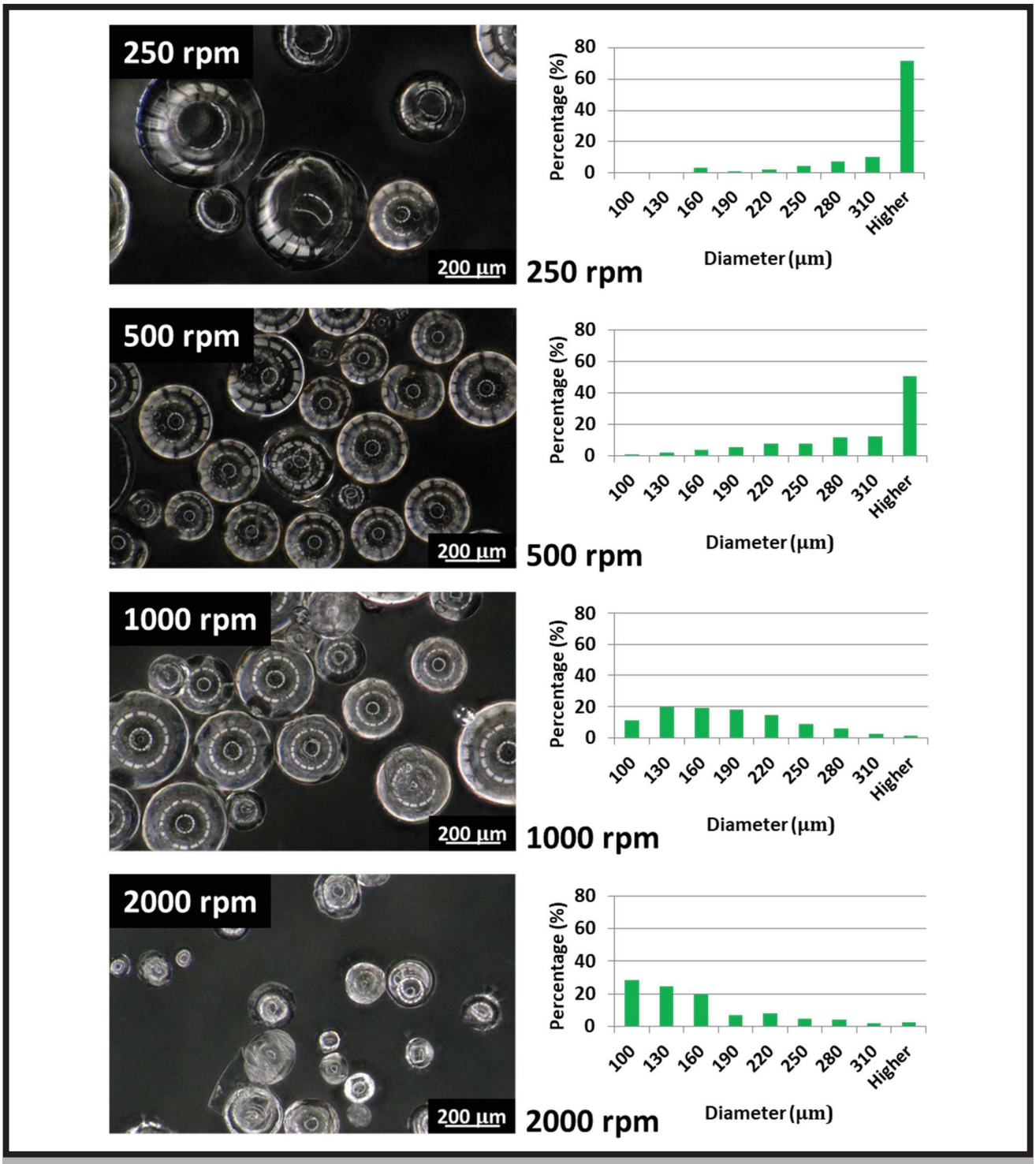

FIG. 2. Optical microscopy pictures (left column) and diameter distribution histograms of PLGA microspheres (right column) obtained at a different stirring speed of water phase (250, 500, 1000 and 2000 rpm); with constant other parameters: PLGA = 7.5%, PVA = 1%; histograms prepared based on n = 600 MS.

PVA concentration in water phase

In this experiment five concentrations of PVA in the water phase were examined: 0.1%, 0.5%, 1%, 2% and 4%. The PLGA concentration in the DCM oil phase was 7.5% and the stirring speed – 1000 rpm. The MS morphology, size and size distribution were analyzed and compared. The results of the MS morphology observed under an optical microscope and the corresponding size distribution values are shown in FIG. 3. The results proved all the MS to be transparent and endowed with a smooth appearance (FIG. 3, left column).

The histograms presented in FIG. 3, right column, show that the percentage of the biggest MS (>260 μm) was the highest (44.4%) for the MS produced at the 0.1% PVA concentration. For the higher PVA concentrations (0.5%, 1% and 2%) no significant trend in distribution was observed – a wide variety of diameters in the samples was measured. For the 4% PVA, as expected, the highest number of MS (26.8%) was in the lowest partition of 70 μm and in every next partition it steadily decreased. According to the obtained results, the PVA concentration in the water phase of 0.5-2% was suitable to produce MS. Thus, the 1% PVA was used to manufacture MS in further studies.

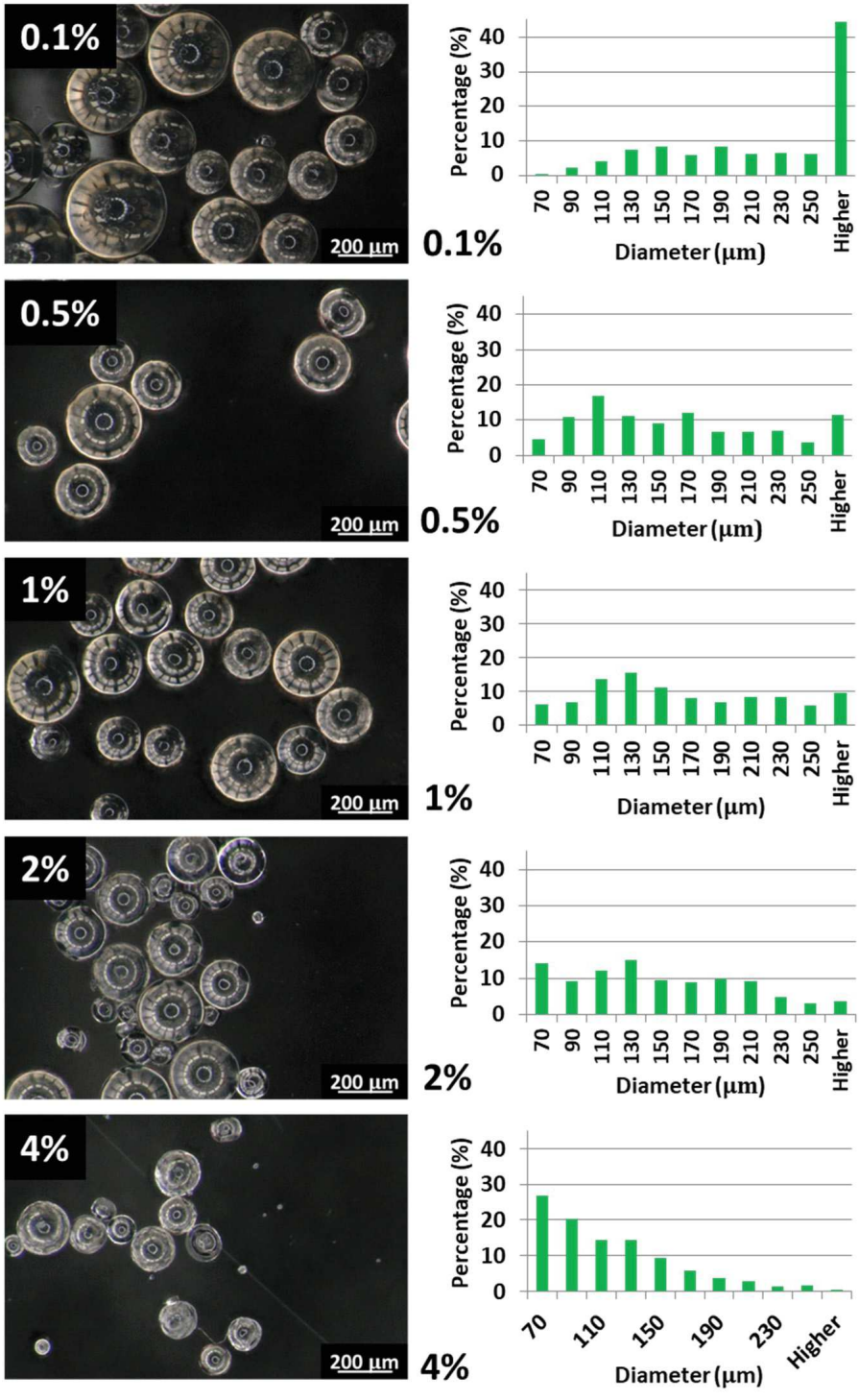

FIG. 3. Optical microscopy pictures (left column) and diameter distribution histograms of PLGA microspheres (right column) obtained at different PVA concentrations in water phase (0.1%, 0.5%, 1%, 2% and 4%); with constant other parameters: PLGA = 7.5%, stirring speed = 1000 rpm; histograms prepared based on n = 600 MS.

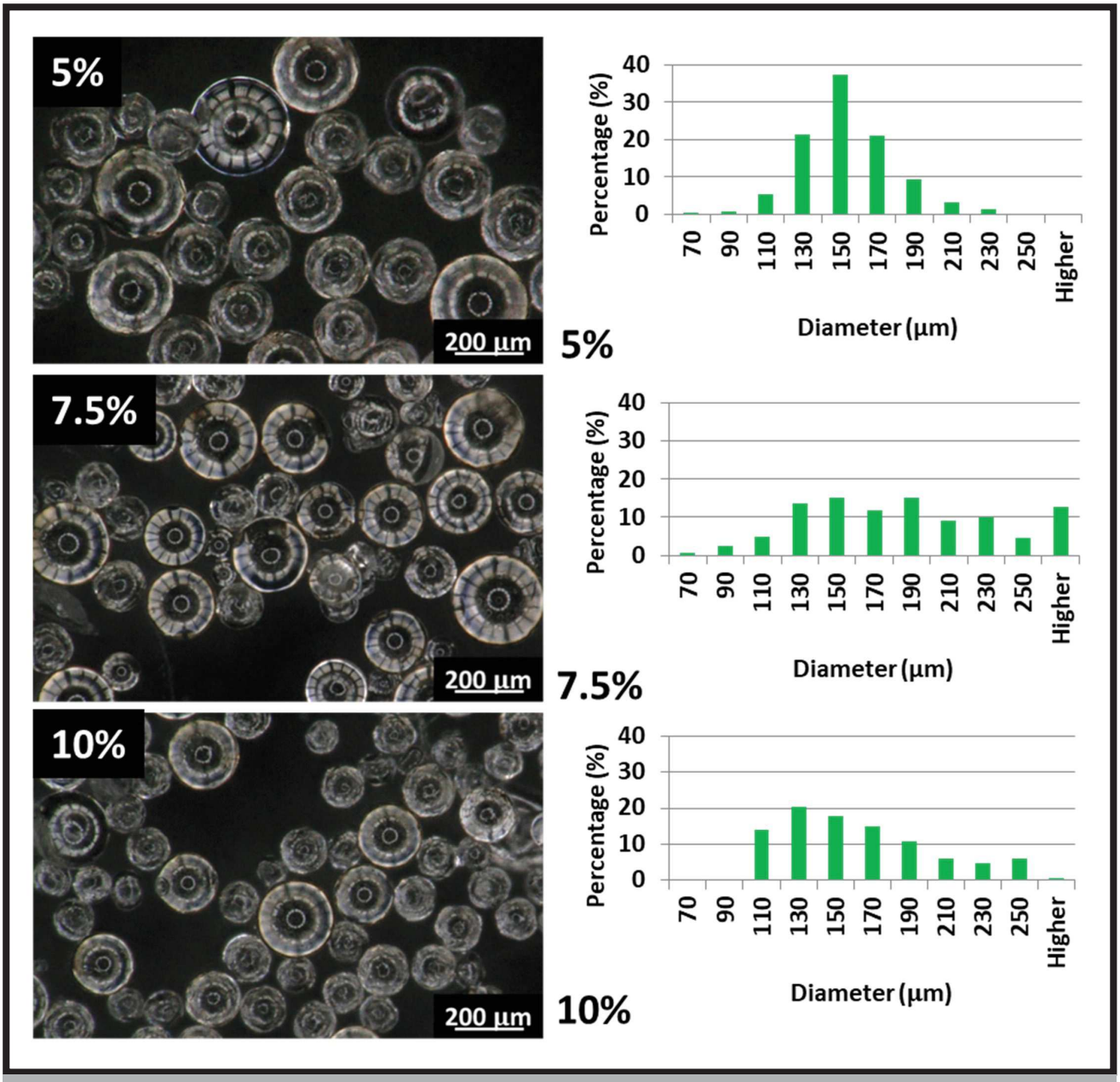

FIG. 4. Optical microscopy pictures (left column) and histograms of diameter distribution of PLGA microspheres (right column) obtained at different concentrations of PLGA in oil phase (5%, 7.5% or 10%); with constant other parameters: 1000 rpm, PVA = 1%); histograms prepared based on n = 600 MS.

PLGA concentration in oil phase

In this experiment three concentrations of PLGA in DCM solution (5%, 7.5% and 10%) were examined and their impact on the MS morphology, size and size distribution was analyzed and compared. In this case, the stirring speed and the PVA concentration were constant (1000 rpm and 1%, respectively). The results showing morphology of the MS observed under an optical microscope and the corresponding size distribution values are shown in FIG. 4. The results showed that all the MS were transparent and exhibited a smooth appearance.

The samples produced from the 5% PLGA concentration in the oil phase revealed the narrowest MS diameter distribution. The highest percentage (37.4%) had the size of 140-160 µm and almost 80% of all the MS obtained at this PLGA concentration fell in the range of 120 µm and 180 µm. However, the obtained data did not show normal distribution (normality checked according to the Shapiro-Wilk test).

The MS diameter distribution was the most irregular at the 7.5% PLGA concentration in the oil phase. These were the only conditions where 12.6% of MS had the diameter higher than 260 µm and for almost 3% of MS the diameter was lower than 100 µm. The MS diameter distribution was more regular for the 10% PLGA concentration than for the 7.5% PLGA in the oil phase. The highest percentage of microspheres (20%) had the size of 120-140 µm. The percentage of MS with higher diameters decreased, for instance less than 1% of all the MS had the diameter higher than 260 µm.

According to the obtained data, the 7.5% PLGA concentration in the oil phase was considered optimal for the MS manufacturing.

Comparison of median MS diameters

In order to visualize and compare the obtained results, median diameters of MS manufactured under different conditions were collected and presented in FIG. 5.

The results shown in FIG. 5A revealed a strong dependence between the stirring speed and the mean MS diameter – namely, the higher the stirring speed, the lower diameter. At the lowest speed (250 rpm) the median diameter was the highest (383.0 µm) and it decreased slightly (to 312.0 µm) when the speed was doubled. The twofold increase in speed (to 1000 rpm) resulted in almost two-time lower median MS diameter (161.5 µm). The further speed increase did not have a considerable impact on the MS median diameter: the 2000 rpm speed led to the MS with the median diameter of 144 µm.

The results presenting the MS median diameter produced at five concentrations of PVA in the water phase (0.1%, 0.5%, 1%, 2% and 4%) are presented in FIG. 5B. The results showed that the PVA concentration significantly influenced the MS diameter: the higher concentration, the lower diameter. The highest median diameter for 0.1% PVA was 323 µm, and the lowest for PVA 4% was 94 µm, so the MS diameter changed more than twice. Therefore, the PVA concentration may be considered a very convenient and effective factor to control the MS diameter.

The results presenting the MS median diameter produced at three concentrations of PLGA in DCM solution (5%, 7.5% and 10%) are presented in FIG. 5C. The results showed that the PLGA concentration in the oil phase did not have a significant impact on the MS median diameter.

In order to perform statistical analysis, we took into account all the data regarding the diameters of 1,800 individual MS. The reason behind such an approach was the fact that the manufacturing conditions shown in FIG. 2 (for the stirring speed of 1000 rpm), FIG. 3 (for 1% PVA) and FIG. 4 (for 7.5% PLGA concentration) were the same and corresponded to the samples obtained at the following parameters: 7.5% PLGA, 1% PVA, 1000 rpm. First, the Shapiro-Wilk analysis revealed that the collected data was not of normal distribution so we presented it as a histogram (FIG. 6). The size distribution proved that the highest number of MS (67.5%) was in the range between 115 and 235 µm. The median diameter of MS equaled 159.4 µm, which almost perfectly fit the expected diameter of 160 µm.

PLGA MS sieving and cleaning process

The optical microscopy pictures and histograms of the PLGA MS diameter distribution before and after the sieving and cleaning processes are presented in FIG. 7. The results showed that MS with more homogenous diameters and diameter distribution were successfully selected thanks to the developed procedure. The sieving and cleaning process effectively increased the MS content with the mean diameter of 280 µm and the MS distribution became more homogenous.

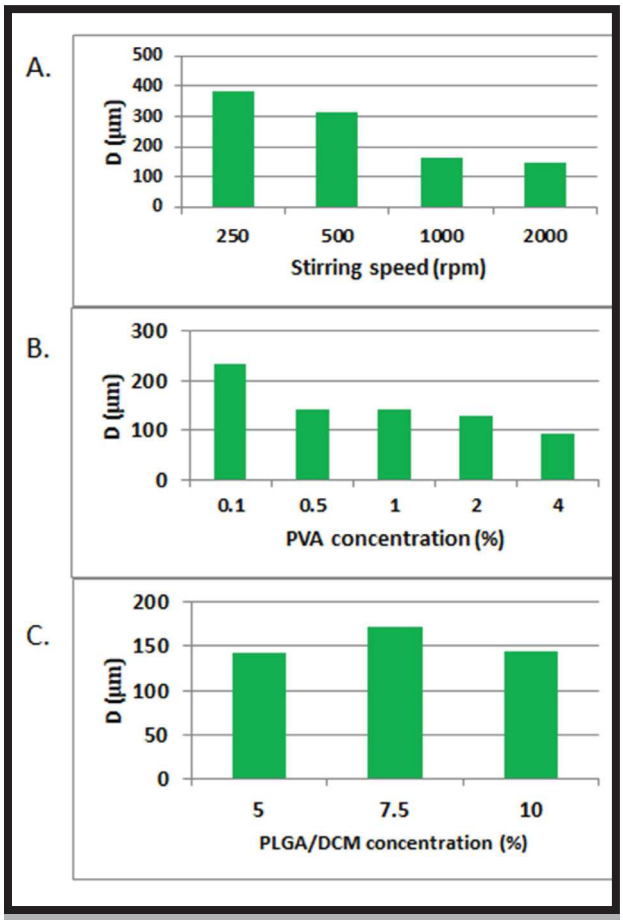

FIG. 5. Median diameter (D) of PLGA microspheres: (A) obtained with a different stirring speed of water phase (250, 500, 1000, 2000 rpm), for constant PLGA concentration of 7.5%, and PVA concentration of 1%; (B) obtained for different concentration of PVA in water phase (0.1%, 0.5%, 1%, 2% and 4%), for constant PLGA concentration of 7.5% and stirring speed in water phase of 1000 rpm; and (C) obtained at different concentration of PLGA in oil phase (5%, 7.5% or 10%); for constant steering speed of 1000 rpm and PVA concentration of 1%; diameter measured for n = 600 individual MS for each manufacturing condition.

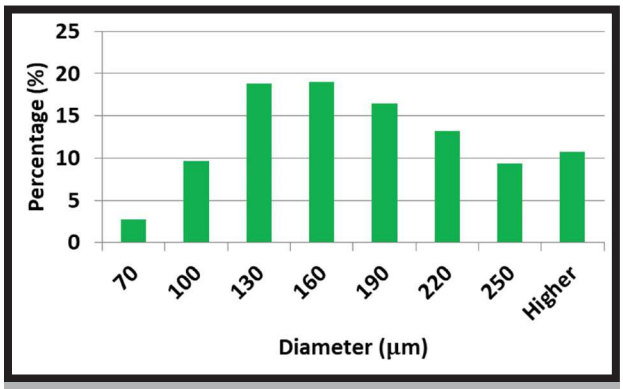

FIG. 6. Histogram of diameter distribution of PLGA microspheres obtained at 7.5% PLGA/DCM concentration in oil phase, 1% PVA concentration in water phase and 1000 rpm stirring speed of water phase; histogram prepared based on n = 1800 MS.

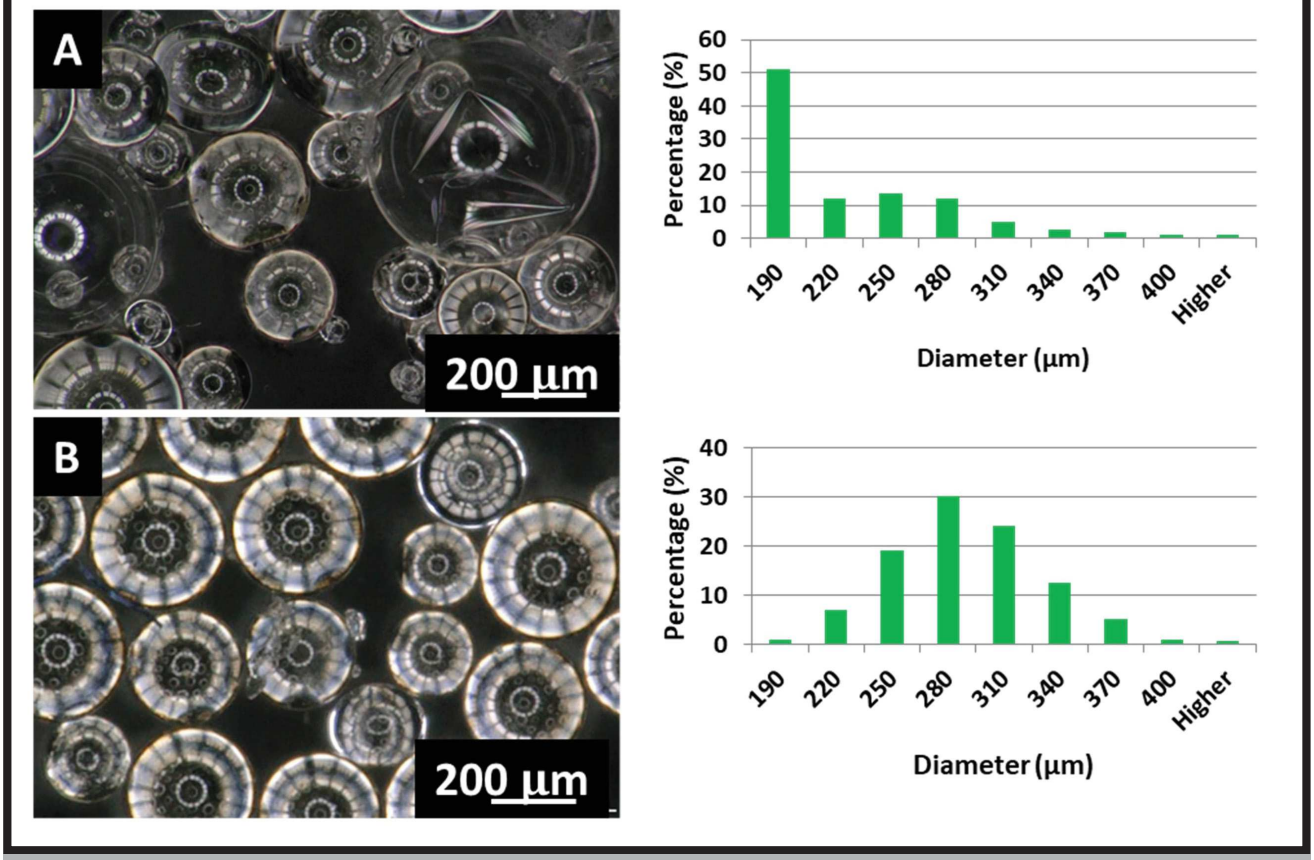

FIG. 7. Optical microscopy pictures (left column) and histograms of diameter distribution of PLGA microspheres (right column) before (A) and after sieving and cleaning process (B) (left column); PLGA concentration in oil phase 7.5%, PVA concentration in water phase 1%, stirring speed 1000 rpm; diameter measured for n = 600 individual MS for each manufacturing condition.

Conclusion

Three parameters – the stirring speed of the water phase, the PVA concentration in the water phase and the PLGA concentration in the oil phase – and their impact on the MS mean diameter and its distribution were examined. The most significant changes were observed for the PVA concentration and the stirring speed – the higher concentration or stirring speed, the lower MS diameter, which was consistent with the literature findings [6].

The aim of our experiments was to obtain MS that could be used for MTE cell cultures and to create cell-tissue constructs. The diameter distribution is of key importance in such an application – the higher variety of diameters is expected to create more complex and irregular constructs. On the other hand, the lower diameter range may be favorable for different applications where high repeatability is desired (e.g. 3D printing with MS used as cell carriers). For all the examined parameters the highest variety of diameters was observed for average values of the investigated PVA concentration in the water phase, the PLGA concentration in the oil phase, and the average values of the stirring speed.

Taking into consideration all the criteria to choose the parameters and the mean diameter, which according to literature should equal 100-300 µm, the optimal manufacturing parameters turned out to be: the 7.5% PLGA concentration in DCM solution as the oil phase, the 1-2% concentration of PVA solution as the water phase and the 1000 rpm stirring speed of the water phase. This combination of parameters guaranteed the MS mean diameter equal to 160 µm.

It was found that due to the MS sieving and cleaning process the MS with more narrow size distribution was successfully obtained. Moreover, only the MS endowed with the mean diameter of 280 µm sank in the cell culture medium and were collected from the cell culture wells bottoms.

All the aforementioned properties are highly advantageous with regard to cell culturing and modular bone tissue engineering applications.

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