MODIFICATION OF PLGA MICROSPHERES' MICRO-STRUCTURE FOR APPLICATION AS CELL CARRIERS IN MODULAR TISSUE ENGINEERING

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Abstract

Microspheres (MS) made of resorbable polymer have been proposed as a cell growth support. They may be assembled to form cell constructs or be suspended in hydrogels allowing injection into injury location. High relative surface area of MS provides more efficient cell culture environment than traditional culture on flat substrates (multiwell plates, Petri dishes). In addition, MS structure, topography and surface chemistry can be modified to promote cell adhesion and proliferation. The aim of this study was to obtain resorbable poly(L-lactide-co-glycolide) (PLGA) MS and to modify their properties by changing manufacturing conditions of the oil-in-water emulsification to better control structural and microstructural properties of MS and their biological performance. To this end, water phase was modified by addition of NaCl to change ionic strength, while oil phase by addition of polyethylene glycol (PEG). Microstructural and thermal properties were assessed. Cytocompatibility tests and cell cultures with MG-63 cells were conducted to verify potential relevance of MS as cell carriers. The results showed that it is possible to obtain cytocompatible MS by oil-in-water emulsification method and to control diameter, porosity and crystallinity of MS with the use of additives to oil and/or water phases without negative changes in MS cytocompatibility. The results prove that modification of both phases make it possible to produce MS with desired/controllable properties like surface topography, porosity and crystallinity.

Keywords: *regenerative medicine, cell cultures, bottom-up, PLGA, emulsification*

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Introduction

Due to high complexity of natural tissues and the need to treat tissue defects and diseases there is a growing interest in tissue engineering (TE) methods. Classical TE, called also top-down TE, uses cells, biologically active molecules and scaffolds to reconstruct tissues *in vitro* or *in vivo*. Conventional scaffolds are macroscopic devices made of different biomaterials which act as artificial extracellular matrices. Scaffolds seeded with cells have a limited ability to mimic natural tissues because they require proper open porosity to let regenerating tissue ingrowth, and cells need nutrients and exchange of many substances to promote angiogenesis, which is essential for wound healing [1]. Modular TE is a new approach called also bottom-up TE, that aims to resolve some problems of conventional top-down TE. Modular TE uses small units like e.g. microspheres, cell sheets, cell aggregates or cell laden modules to build bigger cell-tissue constructs on a way of self-assembly, aggregation or 3D printing [1,2]. This bottom-up TE approach allows to manipulate precisely with cells to combine small elements into more complex tissue systems [3,4].

MS used as cell microcarriers have the main advantage: cells can readily attach to their external surface and relatively high cell density can be achieved after cell culture [1]. Depending on application it is possible to obtain solid or porous MS [5]. Coupling proper porosity and high surface area with growth factors allows to obtain MS with appropriate properties for regenerating tissue [5]. Due to their spherical shape cells can grow in three dimensions [6]. Moreover MS can be suspended in hydrogels and injected into required place in the body [7]. The surface of MS may be modified by other substances like chitosan, fibronectin or collagen to improve cell adhesion and growth [6,8]. Additionally MS can be used to encapsulate drugs and growth factors [8] or be loaded with magnetic particles [9].

Diameter of MS may be controlled by factors like polymer type, properties as well as synthesis method and conditions. Majority of proposed in literature MS for cell growth have diameter between 100-250 μm and are characterized by hydrophilic surface properties [10]. They are usually produced by oil-in-water emulsification. Microspheres size may be controlled by modification of aqueous phase with other substances (e.g. glucose) [9], concentration of polymer in oil phase or stirring speed of water phase [6]. Concentration of emulsion stabilizer, e.g. polyvinyl alcohol (PVA) in water phase also can influence diameter and shape of MS [11]. We hypothesize that modification of ionic strength of water phase by addition of non-toxic NaCl may result in MS with different structure and properties.

The aim of this study was to modify PLGA MS properties by changing manufacturing conditions and composition of oil-in-water emulsification phases to better control structural and microstructural properties of MS and their biological performance. To this end, water phase was modified by addition of NaCl to change ionic strength, while oil phase by addition of polyethylene glycol (PEG) intended to act as a pore former. PLGA being a matrix material was used due to its excellent biological properties and adaptable degradation time, which may be controlled by a ratio of lactide to glycolide and molecular mass.

Materials and Methods

To obtain MS PLGA with molar ratio of L-lactide to glycolide of 85:15, M_n = 100 kDa, M_w = 210 kDa was dissolved in dichloromethane (DCM, Sigma-Aldrich) at a concentration of 20% wt/vol to create oil phase. Water phase was obtained by dissolving 1.5% poly(vinyl alcohol) (PVA) (Mowiol® 4-88, M_w = ~31,000, Sigma Aldrich) in ultra-high quality water (UHQ-water, produced by PureLab, Elga, UK). Three types of MS were obtained: MS1, MS2 and MS3. Reference MS1 were prepared by pouring 1 ml oil phase into 40 ml water phase under stirring on a magnetic stirrer (250 rpm). During injection of oil phase into water phase the tip of the pipette was 0.5 cm above the surface of the water phase. MS2 were produced in the same manner but water phase was supplemented with 0.5% NaCl. For MS3 manufacturing oil phase was modified by addition of 20% PEG (M_n = 400 Da, Sigma-Aldrich). After 24 h the DCM was evaporated and MS were formed, vacuum filtered, rinsed with UHQ-water, dried at 37°C for 24 h, sieved and fraction >100 μm was collected. Manufacturing procedure is shown in FIG. 1.

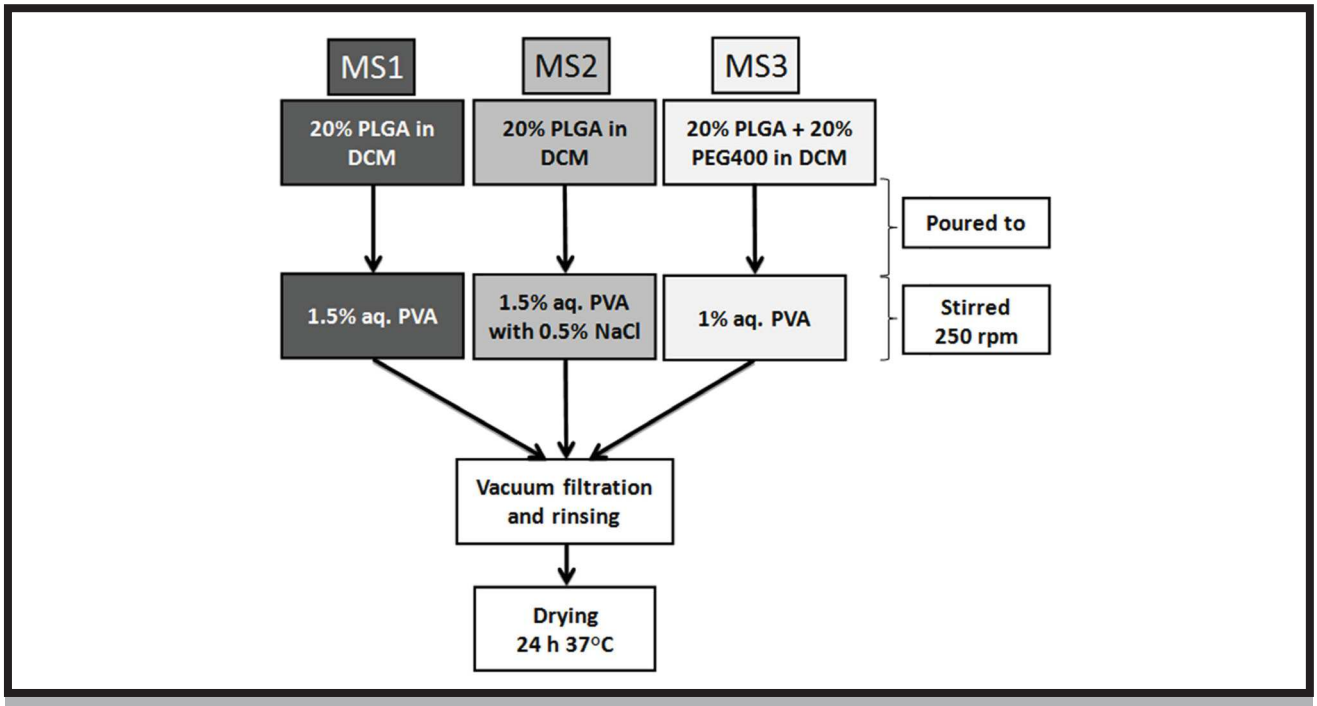

FIG. 1. Manufacturing conditions of PLGA microspheres: MS1, MS2, MS3 by oil-in-water emulsification.

TABLE 1. Microstructural properties of PLGA MS.

Sample	Diameter (mean ±S.D) μm	Diameter (median) μm	Shape factor
MS1	123.0 ±22.0	120.2	1.04 ±0.06
MS2	134.2 ±22.5	132.6	1.03 ±0.06
MS3	127.5 ±21.8	124.8	1.04 ±0.08

MS were analysed with optical microscopes (Axiovert 40, Zeiss and VHX-900F, Keyence), scanning electron microscope (Nano Nova SEM 200) and differential scanning calorimetry (DSC, DSC1 from Mettler Toledo). DSC measurements were performed in the temperature range of -90–200°C at heating rate of 10°C/min in nitrogen atmosphere; sample mass was ca. 6 mg. To measure glass transition temperature (T_g) TOPEM DSC was used with reversing heat flow. To asses size, size distribution and shape factor 300 individual MS were measured from each group with the use of AxioVision software provided with the optical microscope (Axiovert 40, Zeiss).

Before cell culture tests the MS samples were sterilized in ethanol in 48-well plates (Nunclon; 8 mg MS dispersed in 200 μl 70% ethanol was poured into each well). After ethanol evaporation (24 h under laminar hood) MG-63 osteoblast-like cells suspension ($5x10^3$ cells/well) in 500 μl MEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine (PAA, Austria) was added to each well and cells were cultured at 37°C and 5% CO_2. Empty cell culture wells (tissue culture polystyrene, TCPS) acted as control. After 1 and 3 days Alamar Blue assay (Sigma Aldrich) was performed. After 1, 3 and 7 days the cells were stained for live/dead (calcein AM/propidium iodide, Sigma Aldrich) and observed under fluorescence microscope (Axiovert, Zeiss).

Results and Discussion

The aim of this work was to obtain PLGA MS with diameter above 100 μm and with size distribution as narrow as possible and regular spherical shape. Independently from manufacturing parameters average diameter of all microspheres was similar (FIG. 2 A, B, C). MS1 had diameter of 123.0 ±22.0 μm, median 120.2 μm, MS2 had diameter of 134.2 ±22.5 μm, median 132.6, while the diameter of MS3 was 127.5 ±21.8 μm, median 124.8 μm; no significant differences were found according to ANOVA. Also shape factor of all the samples was similar and relatively low (TABLE 1). Distribution of diameters was similar for all the samples (FIG. 2 A, B, C).

Optical microscopy observations (FIG. 2 D, E, F) revealed significant differences between microspheres: MS2 were the most transparent of all examined samples, while MS3 were the most opaque. SEM pictures (FIG. 2 G) showed that MS1 had small pores (few micrometers in diameter) on the surface and were the most rough, while MS2 and MS3 were smooth on the surface (FIG. 2 H, I, respectively).

FIG. 3 shows DSC curves, while TABLE 2 thermal properties of PLGA MS. It is apparent that all MS had similar melting temperature, T_m, ca. 134°C but different melting enthalpy (ΔH_m): MS2 had the lowest ΔH_m, while MS3, the highest. Also glass transition temperature, T_g, of MS2 was the lowest, while for MS3 the highest. The degree of crystallinity, *X*, was calculated from the measured heat of melting, using the value of heat of melting of the crystalline regions of poly-L-lactide, ΔH_m = 93.1 J/g [12], taking into account that in PLGA content of L-lactide was 85%. The results show that the degree of crystallinity of 1.25% was found for MS2, while 6.1% for MS3.

In vitro tests showed that the cells adhered and grew on MS. Observations under fluorescence microscope showed that the cells grew both on TCPS wells and on MS irrespectively of the type of MS (FIG. 2 J, K, L). Majority of the cells were alive (stained green) and only less than 5% cells were dead (stained red). Cell viability after day 1 was significantly different between MS1 and MS2 as well as MS2 and MS3 as shown by Alamar Blue test (FIG. 4).

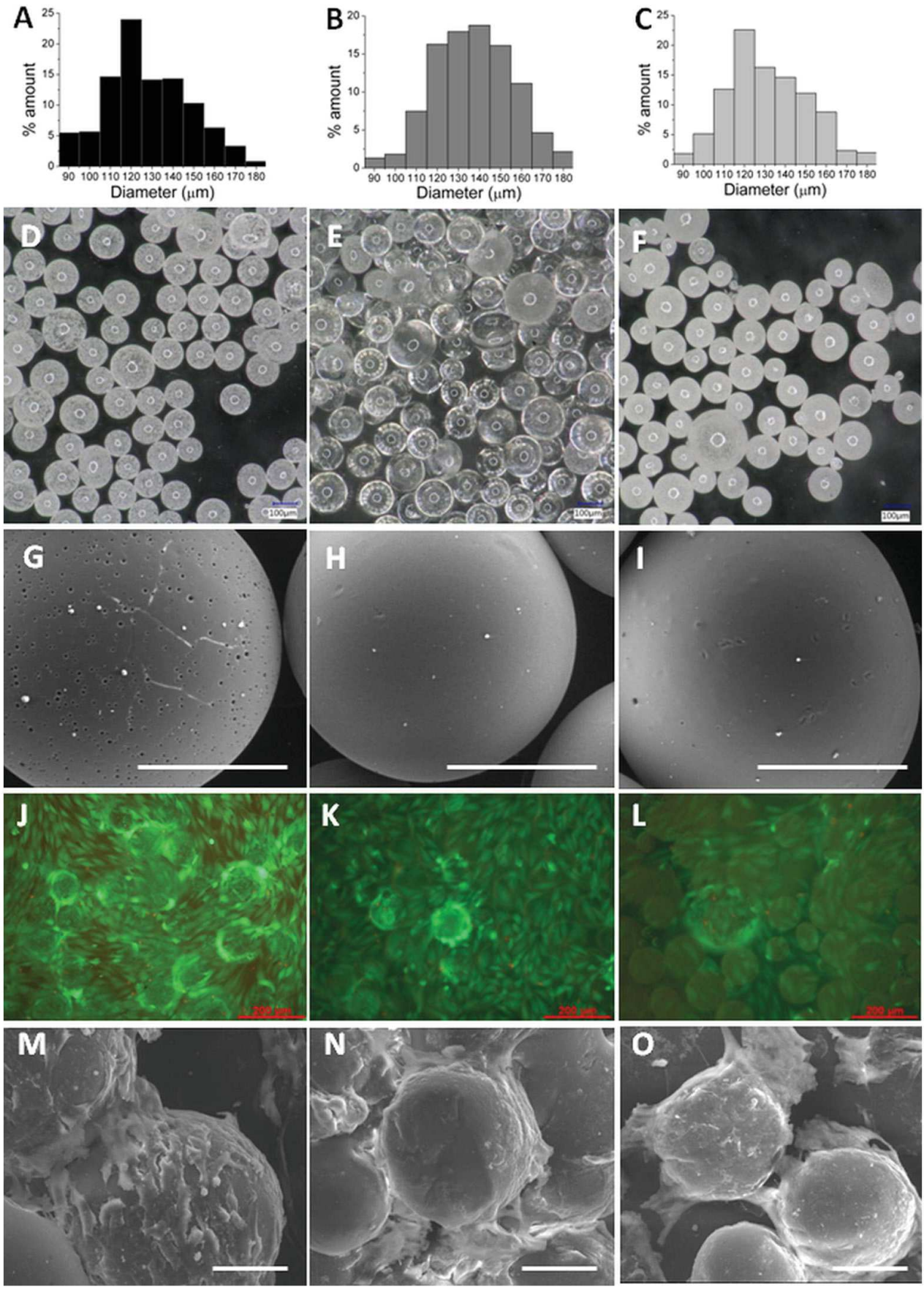

FIG. 2. Histograms of size distribution of MS1 (A), MS2 (B), MS3 (C); optical microscopy images of MS1 (D), MS2 (E), MS3 (F); SEM microphotographs of MS1 (G), MS2 (H), MS3 (I) (scale bar 50 μm); live/dead staining images after 7-day cell culture on MS1 (J), MS2 (K), MS3 (L) and SEM microphotographs of cells after 7-day cell culture on MS1 (M), MS2 (N), MS3 (O) (scale bar 50 μm).

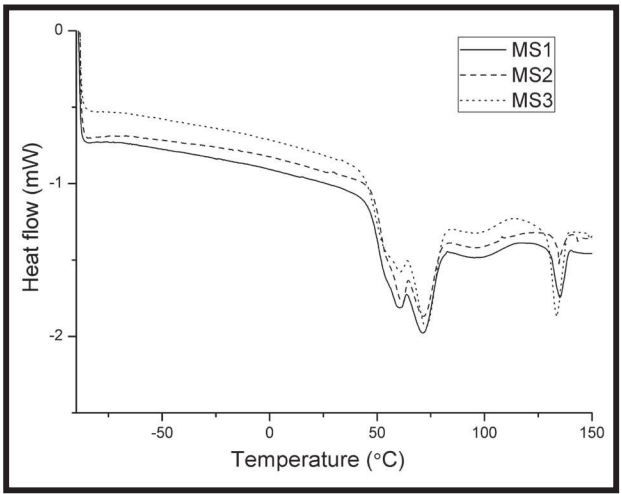

FIG. 3. DSC curves of MS1, MS2 and MS3.

TABLE 2. Thermal properties of MS.

Sample	T_g °C	T_m °C	Melting enthalpy ΔH_m J/g	Crystallinity X %
MS1	54.8	135.09	- 2.47	3.1
MS2	49.0	134.60	- 0.92	1.2
MS3	55.8	133.41	- 4.82	6.1

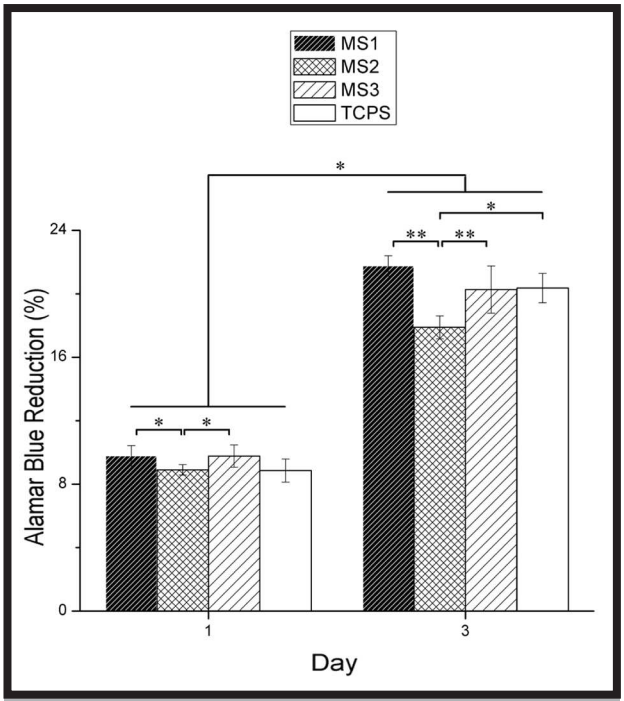

FIG. 4. MG-63 cell viability studied by Alamar Blue reduction on day 1 and 3, no significant differences was found between the samples on the same day, significant difference *p< 0.01, *p< 0.05 according to ANOVA.

On day 3 the viability of cells increased but the lowest viability of cells was on MS2. SEM observations show morphology of the cells cultured on the scaffolds (FIG. 1 M, N, O). It is apparent that the cells adhered well on MPS and in some cases formed monolayers. Some of the cells adhered to two or three MS, as a result the agglomerates were formed.

In this study the aim was to produce PLGA MS by oil-in-water emulsification and to verify if compositions of water and oil phases influence microstructure, thermal and biological properties of resulting MS.

It was found that all MS had the same diameter of 120-130 μm and were of regular shape; shape factor equal to 1.03 – 1.04 was similar to all microspheres. Interestingly, modification of water phase (MS2) and oil phase (MS3) resulted in differences in appearance, transparency, microstructure and thermal properties of the MS. Addition of NaCl to water phase caused high transparency and low porosity of MS2. When oil phase was modified with PEG400 opposite effect was observed: MS3 were opaque. Opacity of MS3 results from higher crystallinity of PLGA as shown by DSC studies – polymer crystallites cause light scattering. Microstructure of MS differed between samples and depended on the used modification approaches but in general the surface of MS was smooth and small pores (few μm in diameter) were found on non-modified MS1.

It was found that modification of water phase with NaCl, which increased its ionic strength, resulted in more amorphous PLGA forming MS. Presence of NaCl, which can be occluded between macrochains of PLGA, had an impact on T_g and crystallinity, what suggests that sodium and chloride ions acted as plasticizer for PLGA, by increasing the distance between polymer microchains. Addition of PEG to PLGA solution in DCM (modification of oil phase) resulted in increase in crystallinity of MS, probably due to the fact that PLGA and PEG did not form a physical mixture but undergo phase separation as shown in our previous study [13]. The second reason might be that PEG chains can interact with PLGA macrochains with hydrogen bonds, which increase T_g, T_m and enhance formation of more organized, crystalline phases. The findings regarding crystallinity are important from the point of view of using MS in cell culture, because it is known that crystallinity influences degradation kinetics of PLGA.

Irrespectively of differences in crystallinity *in vitro* tests proved good cytocompatibility of all investigated MS. Alamar Blue assay showed that the best cell supporting properties were found for MS1 and MS3. Live/dead staining showed that adhesion of cells on MS surface after 7 days was high and a vast majority of the cells were alive (>95%). Additionally SEM microphotographs showed that microspheres with cells tended to form agglomerates what additionally proved high cell compatibility of tested MS.

Conclusions

Obtained results demonstrate that it is possible to obtain microspheres with defined properties like porosity, size and crystallinity by controlling emulsification process parameters with additions of chemicals to oil or water phases. All MS were cytocompatible what allows to consider them as cell carriers in modular tissue engineering.

Acknowledgements

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