

MESENCHYMAL STEM CELLS PROLIFERATION AND OSTEOGENIC DIFFERENTIATION ON POLYMERIC SCAFFOLDS AND MICROSPHERES FOR BONE TISSUE ENGINEERING

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Abstract

In this study, we aimed to compare how the microstructure and architecture of polymer supports influence adhesion, growth and differentiation of human mesenchymal stem cells (hMSC) in the context of bone tissue engineering. We manufactured poly(L-lactide-co-glycolide) (PLGA) three-dimensional supports in the form of microspheres by emulsification and porous scaffolds by solvent casting/porogen leaching. HMSC were seeded on both materials and on control tissue culture polystyrene (TCPS, bottom of the wells) and cultured in basal or osteogenic medium for 1, 3, 7 and 14 days. HMSC proliferation and osteogenic differentiation were studied using lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) assays, respectively. Furthermore, cell morphology and viability were analyzed after live/dead fluorescence staining. The results show that the optimized emulsification conditions allowed the production of PLGA microspheres with a median size of 95 μm. The PLGA scaffolds had a porosity of 82.1% ± 4.2% and a pore size of 360 μm ± 74 μm. HMSC cultured on control TCPS in osteogenic medium were more spread and polygonal than those in basal medium. They were characterized with a lower proliferation rate, as shown by the LDH results, but higher ALP activity. This suggests that hMSC osteogenic differentiation was achieved. The same tendency was observed for cells cultured on microspheres and scaffolds. Cell proliferation was more efficient on both materials and control in growth medium as compared to differentiation medium. The amount of ALP, i.e. a marker of osteogenic differentiation, was elevated, as expected, in differentiation medium. However, on day 14 cells cultured on the scaffolds in basal medium exhibited the same osteogenic potential as those cultured in differentiation medium. In general, both microspheres and scaffolds promoted hMSC adhesion, proliferation, and osteogenic differentiation and may be used for bone tissue engineering.

Keywords: bone tissue engineering, scaffold, microspheres, proliferation, osteogenic differentiation

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Introduction

Bone is a highly specialized tissue, which, due to its structure, can be called a natural polymer-ceramic composite [1,2]. Bone tissue consists of living cells embedded in a matrix that comprises 30% organic substances (collagen, proteins, lipids, proteoglycans, osteopontin) and 70% inorganic substances (mainly hexagonal hydroxyapatite, arranged parallel to the long axis of collagen fibers) [1].

In terms of regeneration, bone tissue is unique because it undergoes complete and continuous regeneration without scarring. Bone trauma is the most common factor inducing the cascade of repair processes. The process of bone tissue regeneration can be divided into three phases: inflammation, bony callus formation, and bone remodelling [1,3]. During that process, the structural composition, cellular composition and biomechanical functions of the pre-injury tissue can be practically completely restored. Nevertheless, there is a small percentage of injuries when healing does not occur as expected. This applies mainly to extensive injuries whose volume exceeds the size of the critical defect. These types of injuries usually require surgical intervention.

Poly(L-lactide-co-glycolide) (PLGA) is a linear copolymer of L-lactide and glycolide. It degrades to lactic acid and glycolic acid, which at higher concentrations are cytotoxic. It is possible to regulate the rate of degradation of PLGA by changing the percentage of each monomer. As a rule, the degradation time is in the range of several weeks to several months [4,5]. This material is commonly used in tissue engineering due to its ease of processing, excellent biocompatibility, good mechanical performance, and degradability.

Scaffolds are three-dimensional porous structures that are permeable to cells and designed for temporary contact with tissues [6,7]. An ideal scaffold should be biocompatible and biodegradable. Over time, the scaffolds should degrade, thus giving space for cells to continue their growth. Scaffolds should provide a space in which the formation of new tissue, neovascularization, or remodelling of the regenerated structure can take place, with the aim of integrating it into the patient's tissues. Several conditions regarding the architecture of scaffolds are necessary for the above-described phenomenon to take place. First of all, the pores should be interconnected so that the diffusion of oxygen and nutrients into the cells and the diffusion of metabolites from the cells is possible [8]. Porosity contributes to better adhesion and migration of cells into the scaffold. To do this, scaffolds should imitate the mechanical conditions and natural bone structures that encourage cell differentiation into the desired phenotype. The optimal porosity is supposed to be within the range of 60% and 90%. Ideally, they would include a degree of micropores with sizes less than 10 μm to promote cell-scaffold interactions and micropores with diameters close to 300 μm to support osteogenesis [7].

Microspheres can be described as small spherical particles (from 1 μm to 1000 μm) that have the ability to move freely [9]. Their main advantage is that they have a relatively large surface area with a small volume, which is why they have quickly become popular in medicine as drug carriers or cell culture substrates [10]. Due to this feature, they provide better access to nutrients and oxygen and at the same time assure efficient metabolic waste removal. Moreover, since microspheres are regarded as 3D constructs, they provide an environment similar to natural conditions in which cells proliferate in living organisms [11].

Another advantage is that there are a large number of methods of obtaining microspheres such as emulsification, coacervation, or phase separation [10]. Mielan et al. obtained PLGA microspheres with an average diameter of $165 \pm 47 \mu\text{m}$. Their research proved that human mesenchymal stem cells (hMSC) proliferated and differentiated towards the osteogenic lineage successfully when cultured on PLGA microspheres [12].

The aim of this research was to fabricate 3D supports made of PLGA in the form of microspheres and scaffolds and to seed on them hMSC in order to access their adhesion, proliferation, and osteogenic differentiation. The outcome provided information concerning the potential of these materials in bone tissue defect healing, which allows us to assess the effectiveness of 3D supports. To the best of our knowledge, the comparison of PLGA scaffolds and microspheres in the context of MSC growth and osteogenic differentiation has not previously been described.

Materials and Methods

Microspheres fabrication

Poly(L-lactide-co-glycolide) (PLGA, 85% L-lactide, 15% glycolide, synthesized in the Polish Academy of Sciences, Zabrze, Poland), poly(vinyl alcohol) (PVA, Mowiol, $M_w = 31,000 \text{ g/mol}$, Sigma Aldrich), and dichloromethane (DCM, PureLan, 99.7%) were used in this study. Microspheres were obtained using the emulsion method with solvent evaporation. The first step was to prepare a 1% PVA solution by dissolving 10.2 g of PVA in 1000 ml of distilled water. In order to obtain a homogeneous solution, it was placed on a magnetic stirrer (Jeio Tech, Multi-Channel Stirrer, MS-52M) at a speed of 400 rpm for 24 h. At the same time, a PLGA solution was prepared by dissolving 1.2 g of PLGA in 30 ml of dichloromethane (DCM). The prepared solution was placed on a magnetic stirrer (400 rpm) for 24 h.

After this time, 50 ml of the previously prepared PVA solution was poured into each of 10 beakers and 3 ml of PLGA solution was added dropwise using an automatic pipette. The beakers were then placed on a magnetic stirrer (400 rpm) for another 24 h. Stirrers of the same size were placed in the beakers so that the movement generated by them was comparable in each beaker. After the solvent (DCM) had completely evaporated and spherical microspheres could be clearly seen in the beakers, they were filtered through filter paper under pressure and rinsed with distilled water. As a next step, the papers with microspheres were placed on Petri dishes and placed in a laboratory dryer at 37°C for 24 h.

Scaffold fabrication

The synthesis of the scaffolds began with the preparation of PLGA solution by dissolving 5.2 g of PLGA in 50 ml of DCM. Then the solution was placed on a magnetic stirrer (400 rpm) for 24 h. Meanwhile, 4.707 g of sodium chloride (NaCl, POCh, Gliwice, Poland) with sieved crystal diameters ranging from 400 to 600 μm were weighed on 10 dishes, into which 5 ml of the previously prepared PLGA solution was poured. The next step in preparing the scaffolds was to mix NaCl, which acted as a porogen, with the PLGA solution using a spatula until the solvent partially evaporated and has the form of a thick "paste". The prepared paste was placed in syringes (5 ml volume), which hubs were cut off and covered with the parafilm. The syringes were left under the fume hood until the solvent evaporated completely. In the next stage, the obtained cylinders were cut into 2 mm thick slices.

In order to completely remove salt, the scaffolds were placed in a beaker with distilled water, which was changed every few hours until the water conductivity stabilized at a constant level on $1 \mu\text{S/cm}$. The whole process took 4 days. After this time, the scaffolds were collected and left to dry on filter paper and then were placed on a Petri dish and left in a laboratory dryer at 37°C until completely dried.

Microscopic observations

Both scaffolds and microsphere morphology were assessed with optical microscopes (Stereo Discovery V8 and Axiovert, both from Carl Zeiss, respectively). Samples were carefully placed on a mirror plate and then pictures were taken. The measurements were performed for 100 pores and 250 microspheres using the ImageJ software. In order to determine the porosity of scaffolds, the sum of the pore areas (measured in ImageJ program) was divided by the area of the photo, and the result was multiplied by 100.

In vitro studies

Human mesenchymal stem cells (ATCC® CCL-1™, American Type Culture Collection, Manassas, VA, USA) were used for cell culture. Both microspheres and scaffolds were sterilized by immersion in ethyl alcohol for 24 h and then exposed to a UV lamp for 30 min. Scaffolds (1 per well) and microspheres (5 mg per well) were placed in a 24-well plate (Avantor, VWR). The culture was carried out at 37°C and at 5% CO_2 . As a next step, the density of the cell suspension was determined so that each well contained the same number of hMSCs (20,000 cells per well). The basal medium (Mesenchymal Stem Cell Growth Medium 2, PromoCell GmbH, Heidelberg, Germany) was added to half of the wells, while to the other half was filled with differentiation medium. It consisted of a basal medium enriched with L-ascorbic acid (300 μM , 1%, Sigma-Aldrich, USA), dexamethasone (10 nM, 0.02%, Sigma-Aldrich, USA) and β -glycerophosphate disodium salt (10 mM, 1%, Sigma-Aldrich, USA).

Live/dead fluorescent staining was used to examine the viability and physiological activity of hMSC cells, by which the presence of live and dead cells could be determined. A solution consisting of phosphate buffer saline (PBS, VWR), propidium iodide (PI, Sigma-Aldrich, Germany), and calcein AM (Sigma-Aldrich, Germany) was used. Both substances were used in the following ratio 1 mg of PI and 1 mg of calcein per 1 ml of PBS. 300 μl of live/dead solution was poured into each well and then incubated for 10 min in a dark place. After 3, 7, and 14 days of cell culture establishment, microscopic observations were carried out using a fluorescence microscope AxioVert 40 (Carl Zeiss, Germany).

Additionally, phalloidin and DAPI staining was performed as to compare the cytoskeletal features of cells in basal and osteogenic medium. First, the medium was removed and the cells were rinsed with PBS enriched with magnesium and calcium ions (Sigma-Aldrich). Then a 3.7% formaldehyde solution (Sigma-Aldrich) was added to the cells for 20 min. As a next step, the cells were rinsed again with PBS solution. In the last step, 0.1% Triton X-100 solution was added to the wells and left there for 5 min. As time passed, the solution was removed and the cells were rinsed with distilled water. After the described procedure, staining dyes were added. First, phalloidin was added to the wells and left in the dark overnight. The following day, the cells were rinsed again with PBS, and DAPI was added. Further steps were the same as for phalloidin. Microscopic observations were carried out using a fluorescence microscope AxioVert 40 (Carl Zeiss, Germany).

LDH and ALP assays

To test lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activity 500 μl of 1% Triton X-100 solution (Sigma-Aldrich) was added into each culture well and then placed on a laboratory shaker (Grant-bio, Sunflower Mini-Shaker, PS-3D, 40 rpm) for 50 min. Meanwhile, a working solution was prepared by mixing 125 μl of solution A (INT/sodium lactate, Takara, Saint-Germain-en-Laye, France) and 5.625 ml of solution B (catalyst (A) diaphoresis/NAD, Takara, Saint-Germain-en-Laye, France). The solution of 0.5M HCl (POCh, Gliwice, Poland) acted as a stop solution.

After 50 min, 50 μl of the contents of each well were poured into a 24-well plate. In the next step, 50 μl of working solution was poured into each well and the samples prepared this way were put away for 8 min in the dark, necessary for the reaction to occur. After this time, 50 μl of stop solution was poured into the wells in order to stop the proceeding reaction. The final step to assess LDH activity was the measurement of absorbance for a wavelength of 492 nm (FluoStar Omega, BMG Labtech, Ortenberg, Germany).

For the ALP assay, exactly the same sample culture plates were used as for the LDH assay. While the plates were on the laboratory shaker for 50 min, a working solution was prepared by dissolving 1 tablet of p-nitrophenol in ALP buffer (0.1M diethanolamine, 0.1% Triton X-100, 1 mM $\text{MgCl} \cdot 6 \text{H}_2\text{O}$, Sigma-Aldrich). 1M NaOH solution (POCh, Gliwice, Poland) acted as a stop solution. After 50 min, 25 μl of the contents of the wells were poured into a 24-well plate followed by 125 μl of working solution. To incubate the samples, they were left for 30 min at 37°C. After this time, 63 μl of stop solution was poured into each well. The final step of the assessment was to measure the absorbance for a wavelength of 405 nm (FluoStar Omega, BMG Labtech, Ortenberg, Germany).

In order to learn precisely about the differentiation potential of cells, the outcome of ALP was divided by cell number so that the results present the activity of ALP osteogenic marker per one cell.

Statistics

Data are presented as an average \pm standard deviation. Statistical analysis was obtained using a one-way analysis of variance (one-way ANOVA) followed by the post-hoc LSD Fisher test using OriginLab software. Probability values lower than 0.05: $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ were considered statistically significant.

Results and Discussions

Microscopic observations

The obtained PLGA microspheres are characterized by a relatively high regularity of shape and a smooth surface (FIG. 1A). In the images, the variation in terms of their size is visible. After analysis of the histogram, it can be seen that the size scatter is almost symmetrical (FIG. 1B). The most frequently occurring microspheres (29% of the total fraction) were in the range of diameters from 90 to 100 μm . Microspheres with diameters in the 80-90 μm and 100-110 μm ranges accounted for 18% of the total each. Microspheres with the smallest diameters (60-70 μm) were the least numerous, accounting for 5.5% of the total and those with the largest diameters, i.e., 120-130 μm (5% of the total) and 130-140 μm (5.5% of the total) ranges. Thus, it can be seen that the diameters of the largest microspheres were practically twice as large as those of the smallest ones. In summary, a large number of medium-sized microspheres in the 80-110 μm range was obtained. The median size of the microspheres was 95 μm .

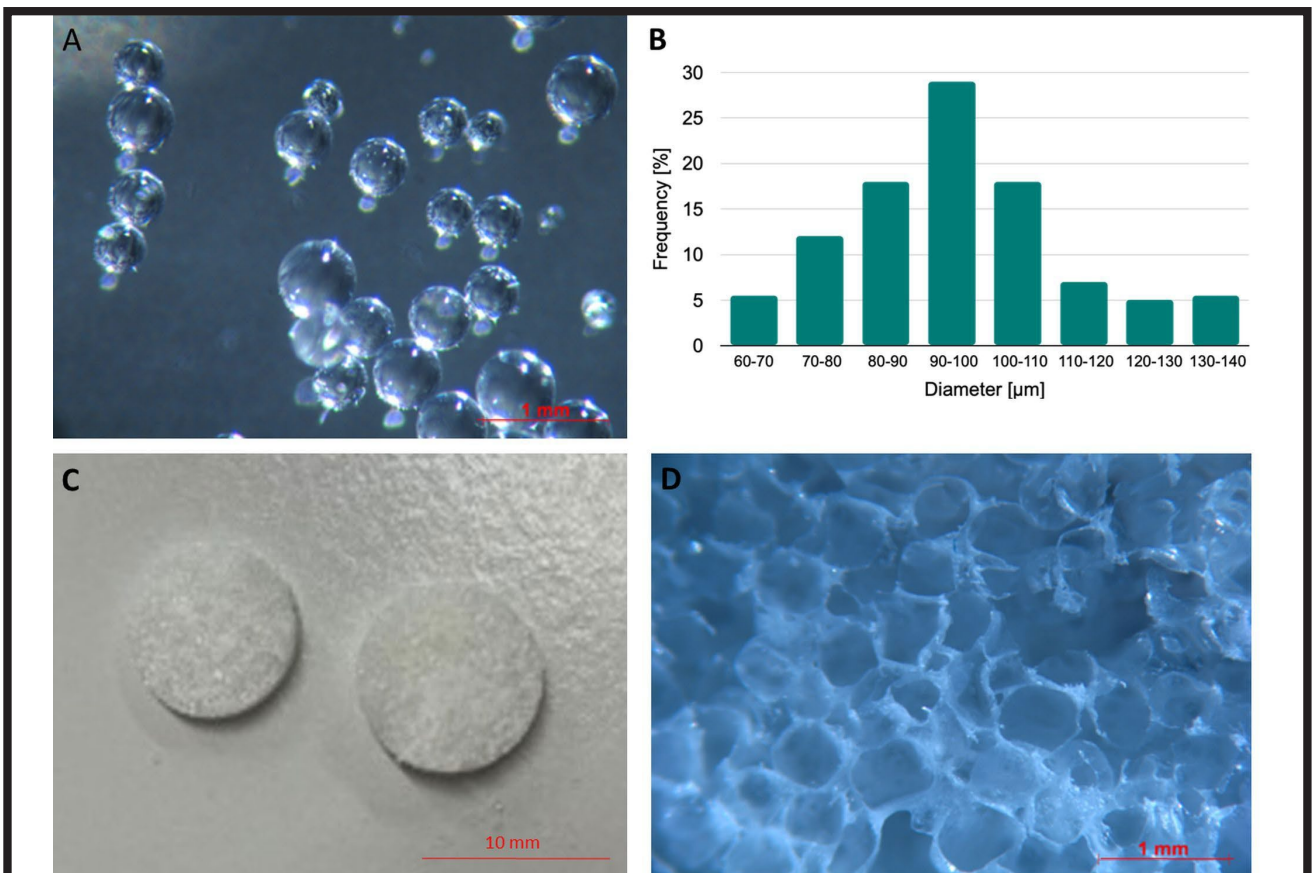


FIG. 1. Morphology (A) and size distribution (B) of PLGA microspheres. Gross morphology (C) and microstructure (D) of PLGA scaffolds. Scale bar (A, D) = 1 mm, (C) = 10 mm.

As shown in FIG. 1C the scaffolds had the form of cylinders 10 mm in diameter and 3 mm in height. Under higher magnification (FIG. 1D), one can observe that they consist of pores with size $360 \mu\text{m} \pm 74 \mu\text{m}$. The porosity of the scaffolds was $82.1\% \pm 4.2\%$. In the scaffolds we can see both concave and convex surfaces. It has been shown that topography and microstructure are very important cues regulating a variety of biological phenomena such as differentiation, morphogenesis, or cell migration [13]. Werner et al. noticed that concave surfaces promote efficient cell migration, whereas convex surfaces promote osteogenic differentiation as convexity leads to cell body flattening [14]. That morphology modification is accompanied by an increase in the level of laminine, which eventually leads to enhanced osteogenic differentiation [13].

In vitro studies

To assess cell morphology phalloidin/DAPI staining and live/dead staining were performed. Already after 7 days significant differences concerning cells shape were noticeable. In differentiation medium cells were more spread and had characteristic polygonal shape, whereas cells cultured in basal medium were more elongated as shown by staining of the cytoskeletal actin fibers (by phalloidin) and nuclei (by DAPI) (FIG. 2A, FIG. 2B). It is also noticeable that the number of cells in basal medium is larger than in differentiation medium. After 14 days it is possible to distinguish certain changes in cell morphology after live/dead staining.

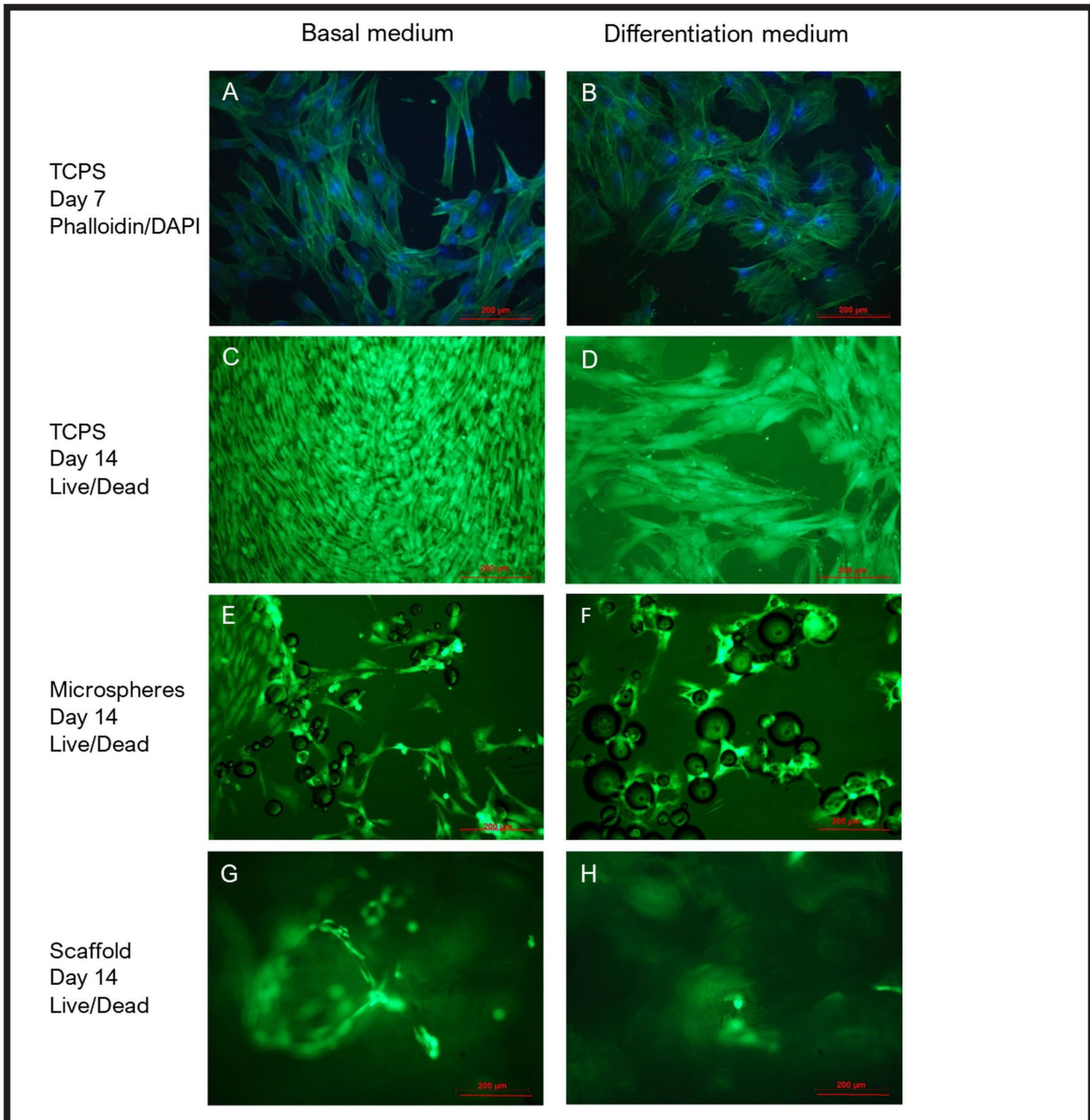


FIG. 2. Fluorescence microscopy images of hMSC cells on: TCPS on day 7 after seeding in basal medium (A) and differentiation medium (B) and on day 14 after seeding in basal medium (C) and differentiation medium (D); microspheres on 14 day after seeding in basal medium (D) and differentiation medium (E); scaffolds on day 14 after seeding in basal medium (G) and differentiation medium (H); phalloidin/DAPI staining (A, B) and live/dead staining (C-H); scale bar 200 μm .

Cells cultured on tissue culture polystyrene (TCPS) in basal medium occurred in larger numbers compared to those cultured in differentiation medium (FIG. 2C, FIG. 2D). The same tendency as regards proliferation was observed for cells cultured on microspheres and scaffolds (FIGs 2E-H). After 14 days of hMSCs culturing, the number of cells in basal medium was much higher than in differentiation medium. Additionally, cells in differentiation medium exhibited distinct morphological characteristics indicative of differentiation towards osteogenic lineage, but their proliferation was reduced. This observation indicates that supplements added to differentiation medium impacted cell behavior as proliferation was reduced due to enhanced osteogenic differentiation.

The results of ALP and LDH assessment confirmed observations described for microscopic studies. As shown by LDH assay (FIGs 3 A,C,E) for all the time points proliferation was more efficient for hMSCs cultured on TCPS, microspheres and scaffolds in basal medium than those cultured in differentiation medium. On day one, the number of cells was similar for all materials in both basal and differentiation media. For the next days, number of cells was gradually increasing. The most dynamic growth of cells was noticed from day 7 to 14 from cell seeding. Cells proliferated similarly on 3D constructs. However, proliferation was always more efficient for cell cultures in basal medium rather than differentiation medium. Similar results were obtained by Rumian et al., as the cells proliferated efficiently on PLGA scaffolds throughout the experiment [8].

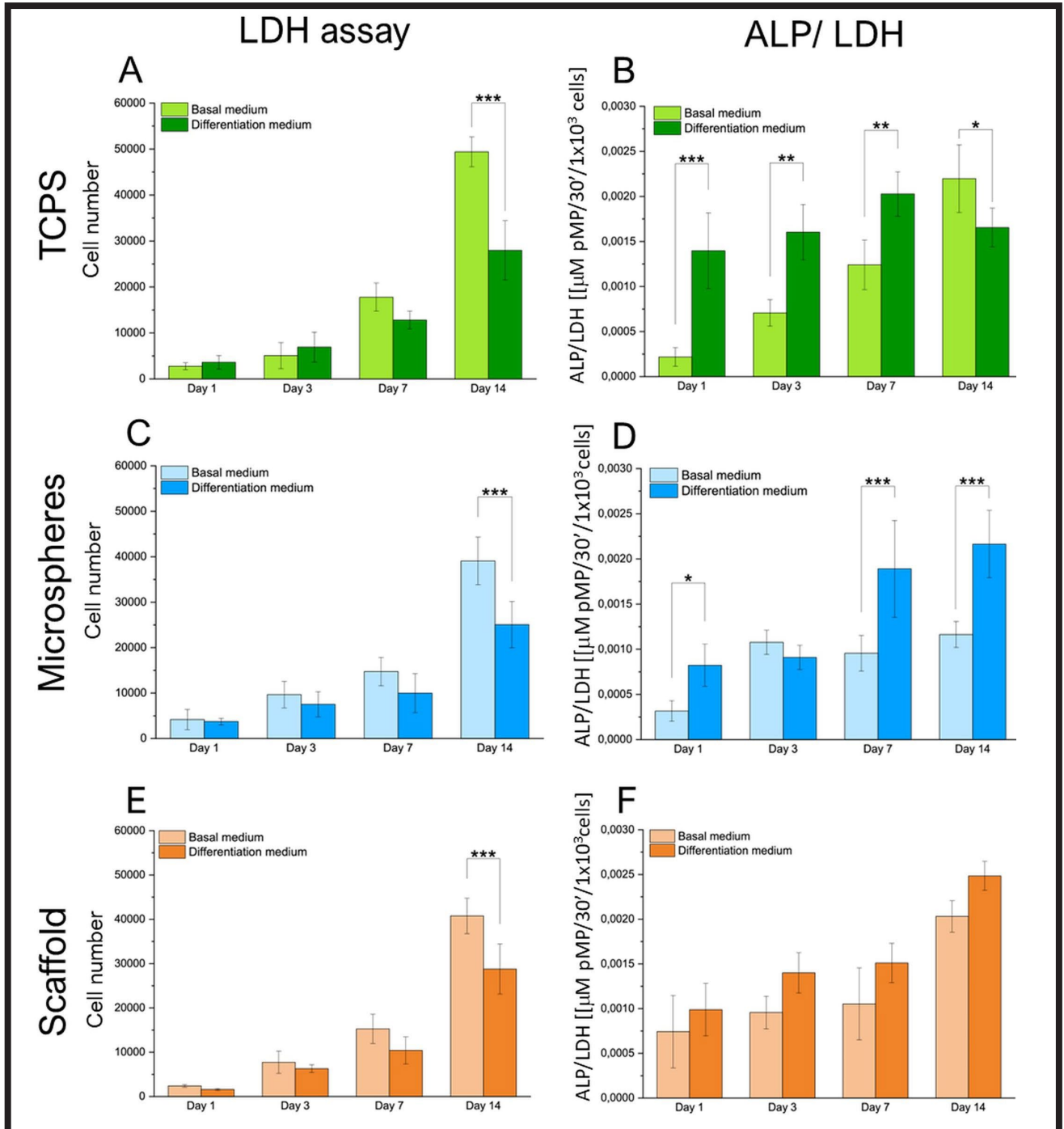


FIG. 3. LDH assay results for: A – microspheres, C – scaffolds, E – TCPS; ALP/LDH results for: B – microspheres, D – scaffolds, F – TCPS, $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ as compared cells cultured in different media. $p^\# < 0.05$, $p^{\#\#} < 0.01$ as compared to cells cultured on TCPS on respective time point.

The ALP activity related to cell number tested by LDH assay was used to evaluate the osteogenic differentiation potential of the hMSC as a function of culture time. In the case of cells seeded on TCPS (FIG. 3B), the ALP activity was rising vigorously in differentiation medium as compared to basal medium up to day 7. On day 14, ALP activity significantly dropped for cells cultured in differentiation medium. For cells cultured on the microspheres (FIG. 3D) amount of this marker was lower than on TCPS. On the scaffolds (FIG. 3F), the ALP activity was the same as on TCPS. This observation indicates that differentiation medium, as expected, promoted cell ALP activity. Nevertheless, for cells cultured on scaffolds on day 14, ALP activity in basal medium was the same as in differentiation medium. It is worth noticing that significant statistical differences between days 1 and 14 for each material were observed. This is a very promising result, suggesting that signals transduced by the microspheres and scaffolds can activate cells for osteogenic differentiation even without supplementing the medium with osteogenic factors. Similar results concerning cell number and ALP expression for hMSC were obtained by Rutledge et al. Both, cell number and activity of ALP increased slowly till day 7, then more dynamic growth was observed. However, the most efficient cell growth was observed for cells seeded on TCPS, whereas more efficient ALP expression was observed for cells seeded on PLGA scaffolds [15].




Conclusions

The PLGA microspheres obtained by emulsification method were non-porous and had a median size of 95 μm . The PLGA scaffolds obtained by the solvent casting/porogen leaching method had a microstructure with porosity of $82.1\% \pm 4.2\%$ and a pore size of $360 \mu\text{m} \pm 74 \mu\text{m}$ that mimics spongy bone. The LDH test proved that more cells on day 14 were present in cultures with basal medium rather than differentiation medium, but cell proliferation was the same on the scaffolds, microspheres and control TCPS. hMSC osteogenic differentiation potential, as expected, was enhanced in differentiation medium. However, for respective time points, cells exhibited higher ALP activity in basal medium when cultured on the scaffolds as on control TCPS. On day 14 cells cultured on the scaffolds in basal medium exhibited the same osteogenic potential as those cultured in differentiation medium. Thus, both microspheres and porous scaffolds were found more suitable for promotion of cell adhesion, proliferation, and differentiation, even when cultured in a medium not supplemented with osteogenic differentiation factors. In clinical conditions, when mechanical support is needed, we recommend implantation of the scaffolds. However, the application of microspheres can be considered when a low-invasive administration of the cell-biomaterial construct is required.

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