POLYMERIC NANOFIBROUS SCAFFOLDS REINFORCED WITH DIAMOND AND CERAMIC NANOPARTICLES FOR BONE TISSUE ENGINEERING

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Abstract

Three types of nanofibrous scaffolds were prepared by electrospining: (1) poly(lactide-co-glycolide) (PLGA) scaffolds reinforced with 23 wt.% of diamond nanoparticles (DNPs), (2) poly(L-lactide) (PLLA) scaffolds with DNPs in concentration ranging from from 0.4 wt.% to 12.3 wt.%, and (3) PLLA scaffolds with 5 wt.% or 15 wt. % of hydroxyapatite (HAp) nanoparticles. The diameter of the nanofibers ranged between 160 and 729 nm. The nanofibers with nanoparticles were thicker and the void spaces among them were smaller. Mechanical properties of the nanoparticle-loaded scaffolds were better, as demonstrated by a rupture test in scaffolds with DNPs and by a creep behavior test in scaffolds with HAp. On PLGA scaffolds with DNPs, the human osteoblast-like MG-63 cells adhered in similar numbers and grew with similar kinetics as on pure PLGA scaffolds. Human bone marrow mesenchymal stem cells grew faster and reached higher population densities on PLGA-DNP scaffolds. However, on PLLA-based scaffolds, the activity of mitochondrial enzymes and concentration of osteocalcin in MG 63 cells decreased with increasing DNP concentration. On the other hand, the metabolic activity of MG-63 cells and content of osteocalcin in these cells were positively correlated with the HAp concentration in PLLA scaffolds. Thus, PLGA nanofibers with 23 wt% of DNPs and PLLA nanofibers with 5 and particularly 15 wt.% of HAp seem to be promising for bone tissue engineering.

Keywords:

Material nanostructure, nanofibers, osteogenic cells, bone implants, regenerative medicine

1. INTRODUCTION

Nanostructured materials, including nanofibrous scaffolds, are important cell carriers for tissue engineering, because they closely mimic the architecture of the native extracellular matrix (ECM), e.g. collagen fibers. In addition, these materials promote adsorption of cell adhesion-mediating molecules (e.g. fibronectin, vitronectin, collagen) from biological fluids (blood, interstitial fluid, cell culture media) in an appropriate geometrical conformation, which enables good accessibility of specific sites in these molecules (e.g. amino acid sequences such as RGD) to cell adhesion receptors, and thus supports the colonization of the material with cells. Among the cell adhesion-mediating molecules, the nanostructured materials adsorb preferentially vitronectin (due to its relatively small and linear molecule), which is preferentially recognized by osteoblasts through the KRSR amino acid sequence in the vitronectin molecule (for a review, see [1]), and thus these materials are particularly important for bone tissue engineering.

However, the nanofibers made of synthetic or natural polymers or their blends are usually too weak for construction of bone tissue replacements, which require high mechanical strength. In the natural bone tissue,

the ECM has two main components: biopolymers and mineral component, represented e.g. by calcium phosphates. Thus, polymeric nanofibrous scaffolds for bone tissue engineering can be reinforced with ceramic nanoparticles, e.g. hydroxyapatite [2], beta-tricalcium phosphate [3] or dicalcium silicate [4]. These nanoparticles not only improved the mechanical properties of the scaffolds, but also increased their bioactivity, manifested by their attractiveness for the adhesion, growth and osteogenic differentiation of cells.

An alternative approach is to reinforce the nanofibrous scaffolds with carbon nanoparticles. For example, electrospun nanofibrous poly-DL-lactide scaffolds were loaded with multiwalled carbon nanotubes (MWCNT). The MWCNT not only created additional nanostructures on the nanofiber surface, but they rendered the scaffolds electrically conductive, which enabled electrical stimulation of osteoblasts and promoted their elongation and directed growth along the electrical current direction [5]. However, carbon nanotubes have been often reported to have adverse effects on cells, such as oxidative stress, resulting in the damage of the cell membrane, mitochondria and cell genome [6]. Similar negative effects have been also reported in fullerenes (for a review, see [7]). Among carbon nanoparticles, diamond nanoparticles appeared as the safest materials. In our earlier studies, nanocrystalline diamond films proved as excellent substrates for the adhesion, growth and osteogenic differentiation of human bone-derived cells [8-10].

Therefore, in this study, diamond nanoparticles (DNPs) were used for reinforcement of nanofibrous PLGA or PLLA scaffolds. Also PLLA scaffolds loaded with hydroxyapatite (HAp) nanoparticles were constructed. The three types of composite scaffolds were then tested *in vitro* with human osteogenic cells in the form of cell lines and primary cultures.

2. MATERIALS AND METHODS

2.1 Preparation and characterization of the scaffolds

The PLGA-DNP scaffolds were prepared from a copolymer of L-lactide and glycolide (ratio 85:5) PURASORB PLG 8531 (Purac Biomaterials, Germany). The polymer was dissolved in a mixture of methylene chloride and dimethyl formamide at a concentration of 2.3 wt%. The ratio of the two solvents was 2:3. The nanofibrous membranes were then prepared by electrospinning in a Nanospider™ machine (Elmarco, Czech Republic) using a vertically-positioned spike-like electrode, on which the polymer solution was applied with a micropipette. Some nanofibrous PLGA membranes were created in combination with DNPs. The concentration of DNPs in PLGA diluted in methylene chloride and dimethyl formamide was 0.7 wt%, and in the pure PLGA after evaporation of the solvents, the concentration was calculated to be almost 23 wt% [11,12].

The PLLA-DNP scaffolds were prepared from poly(L-lactide) (PLLA, Ingeo Biopolymer 4032D) purchased from NatureWorks, Minnetonka, Minnesota, USA. Five grams of PLLA were dissolved in 100 ml of chloroform, and 22 mg to 700 mg of the diamond nanoparticles were added to this solution. The nanofibers were then prepared by needle-less electrospinning, using a Nanospider™ unit (NS Lab 500, Elmarco Ltd., Liberec, Czech Republic). After evaporation of the solvent, the final concentration of diamond nanoparticles in the PLLA ranged from approx. 0.4 wt% to approx. 12.3 wt%.

The PLLA-HAp scaffolds were prepared from the same PLLA as the PLLA-DNP scaffolds. For the electrospinning process, 7 wt.% solution of PLLA in a mixture of chloroform, dichloroethane and ethyl acetate was used. First, the polymer was diluted only in chloroform, and after the dilution, other two solvents were added. To prepare composite PLLA-HAp scaffolds, two concentration of BABI-HAP-N100 (Berkeley Advanced Biomaterials Inc., San Leandro, CA, USA), were added to the prepared PLLA solution before electrospinning. The content of HA in the final dry scaffolds was calculated to be 5 wt% or 15 wt% relative to the polymer. Nanofibers were prepared by a needleless electrospinning technology using a Nanospider™ unit (NS Lab 500, Elmarco Ltd., Liberec, Czech Republic) [13].

Before electrospinning process, both DNP and HA nanoparticles were homogeneously dispersed in the polymer solution by intensive vortexing and sonication in order to minimize clustering of the particles.

The morphology of the scaffolds was characterized by scanning electron microscopy (SEM). The mechanical properties were evaluated by rupture tests of load and deflection of rupture probe at failure [11,12] and the creep behavior of the scaffolds in their dry and wet state [13].

2.2 Cell cultures on the scaffolds

The nanofibrous membranes were detached from the underlying polypropylene substrate, cut into square samples, fixed in CellCrown inserts (Scaffdex, diameter 1.1 cm), sterilized by gamma irradiation or ethylene oxide gas (PLGA-based membranes) or by 70% ethanol for 1 h followed by rinsing in distilled and deionized water and in cell culture media (PLLA-based membranes). The scaffolds were then inserted into polystyrene 24- or 12-well cell culture plates and seeded with human osteoblast-like MG-63 cells (approx. 17,000 cells/cm^2). The PLGA-ND scaffolds were also seeded with human bone marrow mesenchymal stem cells (hMSC, approx. 6,000 cells/cm^2). MG-63 cells were cultured in DMEM medium supplemented with 10% of fetal bovine serum (FBS) and 40 μg/ml of gentamicin [11,13]. For hMSCs, α-MEM medium supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mmol/L *L*-glutamine was used [12]. The cell number, shape and the size of cell spreading area were evaluated by counting cells on microphotographs. Cells were also counted in a Bűrker haemocytometer after trypsinization [11,12]. The cell metabolic activity, i.e. activity of mitochondrial enzymes, was measured by XTT test. The concentration of osteocalcin, a marker of osteogenic cell differentiation, was measured in cell homogenates, obtained by sonication of cells, using an enzyme-linked immunosorbent assay (ELISA) [13].

3. RESULTS AND DISCUSSION

3.1 Cell behavior on PLGA-DNP membranes

On day 1 after seeding, human osteoblast-like MG-63 cells adhered to both pure and DNP-containing membranes in similar numbers and with similar cell spreading area. Also on day 3 and 7 after seeding, the numbers of MG-63 cells were similar on both types of membranes, i.e. the cells proliferated with similar kinetics (Fig. 1 A-C). The cells on both types of membranes were polygonal or spindle-like in shape, were distributed homogeneously on the samples, and on day 7, they were able to form a confluent layer (Fig. 1B, C). However, the hMSC reached significantly higher cell numbers on days 5 and 9 after seeding on the PLGA-DNP membranes (Fig. 1 D-F). This can be explained by a different morphology and better mechanical properties of PLGA-DNP membranes. As revealed by SEM, DNP-loaded PLGA fibers were thicker (diameter 270 ± 9 nm) than pure PLGA fibers (diameter 218 ± 4 nm), and the void spaces among these fibers were smaller (0.46 ± 0.02 μm^2) than among the pure PLGA fibers (1.28 ± 0.09 μm^2). PLGA-DNP membranes also contained more numerous materials clusters (33,361 ± 6,182/mm^2 *vs*. 8,340 ± 1,495/mm^2 in PLGA), which was probably due to imperfect dispersion of DNP in the polymeric matrix (Fig. 2 A, B). The projected area of these clusters was larger in PLGA-DNP (3.54 ± 0.90 μm^2) than in PLGA samples (2.13 ± 0.75 μm^2). In addition, the PLGA-DNP membranes showed higher mechanical resistance, as revealed by rupture tests of the load and deflection of the rupture probe at failure (Fig. 2 C, D). Thus, DNP-loaded membranes might provide a stronger growth support for cells, and primocultured hMSC were more sensitive to the properties of this support than the MG-63 line cells, which are well-adapted to the *in vitro* cultivation [11,12].

3.2 Cell behavior on PLLA-DNP membranes

Surprisingly, the PLLA-DNP membranes did not provide so good growth support for the adhesion and growth of MG-63 cells as PLGA-DNP membranes. On day 3 after seeding, the mitochondrial activity of the cells, which is an indirect indicator of their number and viability, showed a decreasing tendency with the increasing concentration of diamond nanoparticles. The lowest mitochondrial activity was detected in cells cultured on PLLA nanofibers with the highest concentration of DNPs (0.7g DNPs/100 ml of PLLA solution, i.e. about 12.3 wt. % of DNPs in the dry scaffolds (Fig. 3 A). It should be pointed out that this concentration

was almost 2 times lower than the concentration of DNPs in the PLGA scaffolds, which supported the adhesion and growth of MG-63 cells and even increased the performance of hMSC. Similar results were obtained from fluorescence staining of the cells, where the cell population density decreased with increasing concentration of diamond nanoparticles (Fig. 3 B, C). Also the concentration of osteocalcin in the cells, i.e. a late marker of osteogenic differentiation, decreased with the increasing concentration of DNPs in the PLLA nanofibers (Fig. 3 D).

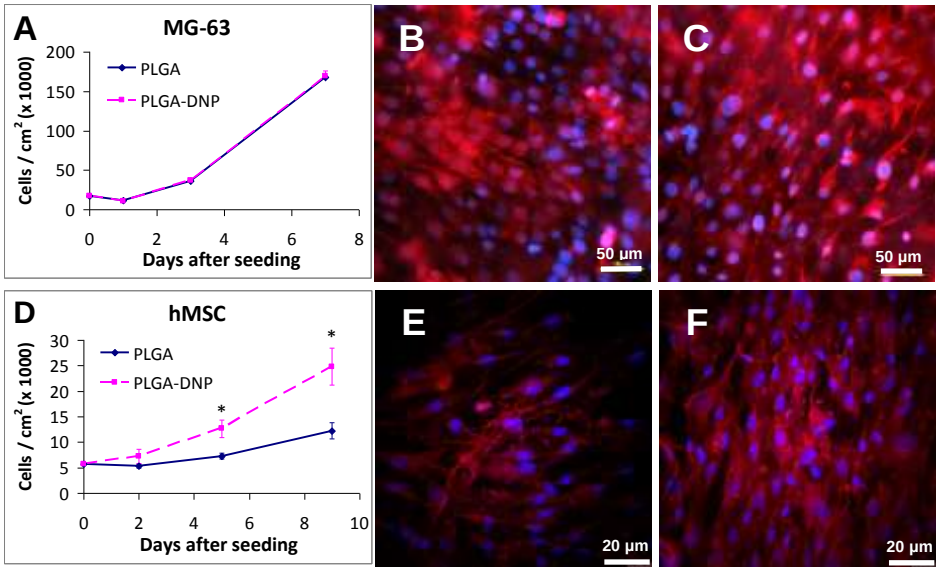

Fig. 1. Growth curves (A, D) and morphology (B, C, E, F) of human osteoblast-like MG-63 cells (A-C) and human mesenchymal stem cells (hMSC, D-F) after seeding on pure PLGA (B, E) and PLGA-DNP scaffolds. A, D: Mean ± S.E.M. (Standard Error of Mean) from 72 measurements (A) or 10-28 measurements (D) for each experimental group and time interval. ANOVA, Student-Newman-Keuls Method. Statistical significance: *p≤0.05 compared to PLGA. B, C: day 7 after seeding, E, F: day 9 after seeding.

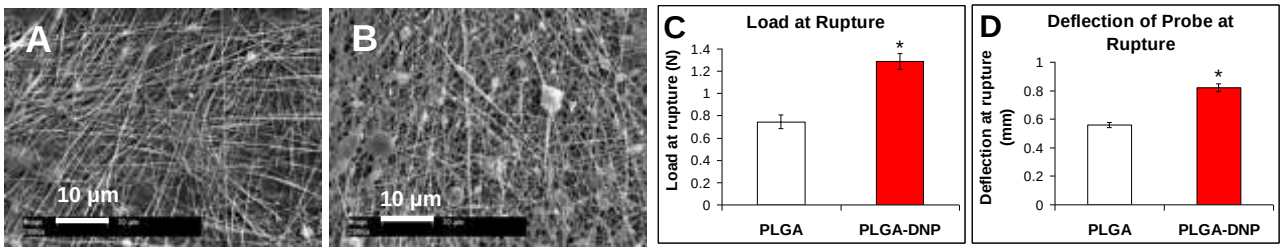

Fig. 2. SEM morphology (A, B) and mechanical properties (C, D) of PLGA and PLGA-DNP scaffolds. C, D: Mean ± S.E.M. from 10 samples for each experimental group. Two-tailed t-test, *p≤0.05 compared to PLGA.

One explanation for these less favorable findings could be a release of DNPs from the PLLA scaffolds into the cell culture medium, their penetration into the cells and adverse effects on the cells. During trypsinization of cells for their counting or homogenization, we observed a grayish color of the trypsin solution, which suggest the release of DNPs into this solution. Some genotoxic and cytotoxic behavior of DNPs suspended in cell culture media has been recently reported, although these adverse effects were milder than in other carbon nanoparticles, such as nanotubes or fullerenes. The adverse effects have been explained by the generation of reactive oxygen species by DNPs [14], and also by an excessive delivery of sodium ions adsorbed on DNPs into cells [15]. The use of different solvents for PLGA and PLLA and different electrospining process (i.e. using needle and needle-less) might also influence the release of DNPs from the fibers and their potential cytotoxicity.

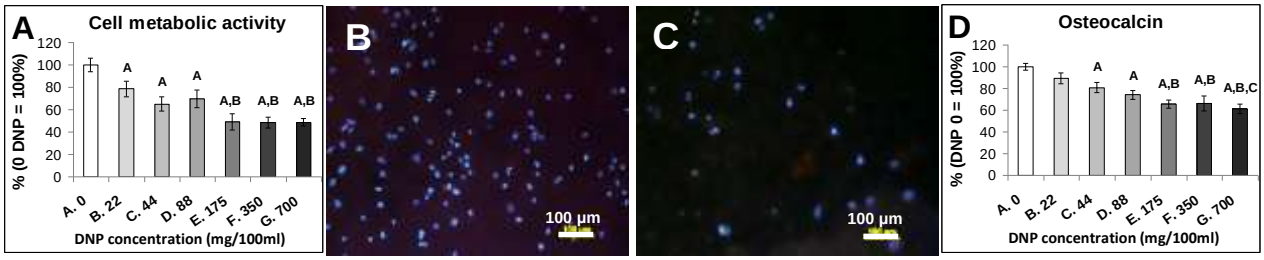

Fig. 3. Metabolic activity (A), morphology (B, C) and concentration of osteocalcin (D) in human osteoblast-like MG-63 cells on day 3 (A-C) and 7 (D) after seeding on PLLA scaffolds with DNP concentration ranging from 22 to 700 mg of DNPs per 100 ml of PLLA solution. B: pure PLLA scaffolds, C: scaffolds with 700 mg of DNPs/100 ml of PLLA solution. Mean ± S.E.M. from 5-6 measurements for each experimental group. ANOVA, Student-Newman-Keuls Method. Statistical significance: ^{A,B,C}: p≤0.05 in comparison with groups labeled with the same letters.

3.3 Cell behavior on PLLA-HAp membranes

On day 7 after seeding, the metabolic activity of MG-63 cells on nanofibrous membranes with 15 wt.% of HAp was significantly higher than on the pure PLLA scaffolds and scaffolds with 5 wt.% of HAp. The cells on samples with 15 wt.% of HAp showed the brightest immunofluorescence of osteocalcin, and also concentration of osteocalcin, measured by ELISA in homogenates of cells detached from these samples, was significantly higher than in cells on pure PLLA samples (Fig. 4). Moreover, the addition of HAp suppressed the creep behavior of the scaffolds in their dry state. Thus, nanofibrous PLLA scaffolds have potential for bone tissue engineering, particularly those enriched with 15 wt % of HA [14].

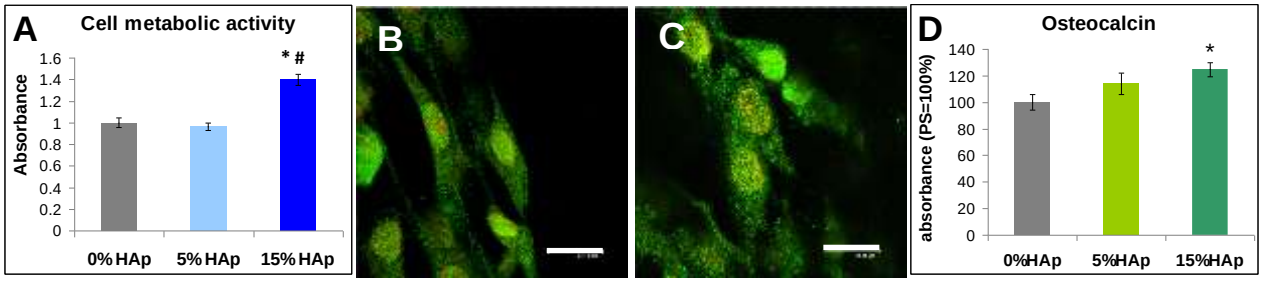

Fig. 4. Metabolic activity of MG 63 cells on PLLA nanofibrous scaffolds with 0, 5 and 15 wt.% of HAp (A), immunofluorescence staining of osteocalcin in these cells on pure PLLA scaffolds (B) and scaffolds with 15 wt.% of HAp (C) and concentration of osteocalcin in cell homogenates (D). A, D: Mean ± S.E.M. from 6 measurements for each experimental group. ANOVA, Student-Newman-Keuls Method. Statistical significance: $^{*, \#}$: p≤0.05 in comparison with PLLA with 0 wt.% and 5 wt.% of HAp, respectively.

CONCLUSION

Nanofibrous PLGA scaffolds enriched with diamond nanoparticles and PLLA scaffolds with hydroxyapatite nanoparticles provided good support for the adhesion, growth and osteogenic differentiation of human osteoblast-like MG 63 cells and human bone marrow mesenchymal stem cells. However, on PLLA scaffolds with diamond nanoparticles, the metabolic activity and concentration of osteocalcin decreased with increasing nanoparticle concentration, which needs further investigation.

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