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Nanophase Alumina/Poly(L-Lactic Acid) Composite Scaffolds for Biomedical Applications

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Abstract

Three-dimensional composites of nanophase alumina and poly(L-lactic acid) with an interconnected porous network and an overall porosity in excess of 90% are cytocompatible. Osteoblast proliferation on the nanophase ceramic/polymer composites is a function of time of cell culture and of nanoceramic loading in the biomaterial substrates.

Introduction

Biomaterials are an integral part of biomedical applications, such as tissue engineering and prosthetic devices, that constitute alternative strategies in addressing the increasing clinical need for replacement tissue. Such materials also provide the potential for alleviating the limitations of autologous tissue availability and the medical problems associated with allografts.

Among the most promising recent biomaterial developments, nanophase ceramics and their composites with polymers have been shown to exhibit selectivity for, and promoted enhancement of, osteoblast (the bone-forming cell) functions pertinent to new bone tissue formation.[1-6] To date, however, the potential of these novel material formulations has only been explored using essentially two-dimensional substrates. Because of the three-dimensional nature of native tissues, and of the pertinent construction requirements for tissue engineering applications, the current study focused on the preparation, characterization, and cytocompatibility of three-dimensional nanophase alumina/poly(L-lactic acid) composite scaffolds in an effort to provide improved material formulations for biomedical applications.

Materials and Methods

Scaffold Preparation Three-dimensional, porous composite scaffolds were prepared using a thermal phase separation process according to established methods.[7] Briefly, poly(L-lactic acid) (PLLA) with a molecular weight of 100,000 (Polysciences) was dissolved in 1,4-dioxane (Sigma-Aldrich) by magnetic stirring at 70 °C for two hours to obtain a 5% (w/v) solution. Appropriate amounts of either nanophase alumina (grain size of 38 nm; Nanophase Technologies) or micron-size alumina (grain size of 1 μm; Sigma-Aldrich) were blended into the polymer solution by vortexing and then stirring at 70 °C for one hour to obtain 50/50, 60/40, 70/30, or 80/20 (w/w) percent ceramic/polymer composites. Each composite was frozen at –20 °C for 2 hours and then at –70 °C overnight, before being freeze-dried at -98 °C and 7 mTorr for 48 hours (to remove the dioxane). All scaffolds were stored in a dessicator for a maximum of 7 days prior to use. Pure PLLA scaffolds were constructed similarly, but without the addition of ceramic, and were used as controls.

Scaffold Architecture and Properties Scaffolds (12.5 mm in diameter and 25.0 mm in height) were cut in half along their diameter, sputter-coated with gold, and visualized using a field-emission scanning electron microscope (JEOL JSM-6330F). Pore architecture and size were determined from measurements on random fields of selected low magnification (x50) SEM images. Porosity was measured on a minimum of 5 scaffolds of each type using a helium pycnometer (Micromeritics AccuPyc 1330). Compression properties were determined using a United SSTM-1-PC testing machine according to established protocols.[8] Scaffold degradation was examined in the absence of cells under static conditions, in phosphate buffered saline, in a humidified, 37 °C, 5% CO_2/95% air environment, and was quantified by determining weight loss over a four week period.

Osteoblast Isolation and Culture The cytocompatibility of the materials examined in this study were investigated using an *in vitro* model. For this purpose, osteoblasts were isolated from neonatal rat calvariae according to established protocols and characterized by alkaline phosphatase activity, synthesis of collagen, and accumulation of calcium in their extra-cellular matrix.[9] These osteoblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (P/S, Gibco) under standard cell culture conditions (a static, humidified, 37 °C, 5% CO_2/95% air environment). The medium was changed every other day.

Osteoblast Seeding and Proliferation on Scaffolds Osteoblasts (260,000 cells) of population number three were seeded onto each scaffold (10x10x3 mm^3) under a 50 Torr vacuum for 5 minutes, followed by the addition of 1 mL of fresh medium. The initial distributions of cells throughout the scaffolds were confirmed after 16 hours by washing the scaffolds three times with phosphate buffered saline (PBS), fixing the cells in methanol for 30 min, staining the cells with Ethidium Homodimer – 1 (Molecular Probes Inc.), cutting the scaffold into consecutive 20 µm sections with a cryotome (Richard-Allan Sci. Microm MH 505 E), and visualizing the cells using fluorescence microscopy. Osteoblast proliferation was monitored at 3, 7, and 14 days. At these times, each scaffold was washed three times in PBS, mechanically pulverized, and then treated with 1 mL of a papain solution (0.125 mg/mL in phosphate buffered EDTA with 10 mM cysteine) at 60 °C for 16 hours (to release the DNA from the cells). Cellular and synthetic material debris were removed by centrifugation at 3,000g at room temperature for 10 minutes. The extracted DNA present in each supernatant was then labeled with Hoechst 33258 (Bio-Rad) and quantified using a fluorometer (TD-360; Turner Designs) according to manufacturer's instructions.

Results

Scaffold Architecture and Properties All scaffolds tested, namely the pure PLLA, 50/50 micron-size alumina/PLLA composites, and 50/50 nanophase alumina/PLLA composites, exhibited similar pore architecture (Figure 1). These pores were irregular in size (average diameter about 150 µm), shape and orientation, and had a high degree of interconnectivity throughout each scaffold. The porosity of the scaffolds was in excess of 90%. The PLLA scaffolds exhibited a significantly ($p < 0.05$) higher porosity than either the micron or nanophase 50/50 composite scaffolds; these composite scaffolds had similar porosities (Table 1). Compression modulus and yield strength for the pure PLLA scaffolds were significantly (p <

0.05) lower than those of either the micron or nanophase 50/50 composites. The mechanical properties of the micron and nanophase 50/50 composites tested were similar (data not shown). None of the scaffolds tested during the present study exhibited weight loss over the four week period examined (data not shown).

Osteoblast Distribution As illustrated by the micrographs shown in Figure 2, the technique used to seed the osteoblasts resulted in the distribution of viable cells throughout the three-dimensional scaffolds tested at early times (up to 16 hours) of culture. However, beyond 24 hours and under the static cell culture conditions used for these experiments, osteoblasts in the center of the scaffolds did not survive. In contrast, cells present in the outermost (approximately 500 μm from the nearest surface) region of each scaffold remained viable for all durations (up to 7 days) tested (data not shown).

Cytocompatibility As evidenced by the results of the osteoblast proliferation experiments (Figures 3 and 4), all materials tested in the present study were cytocompatible. In fact, compared to results obtained on pure PLLA scaffolds, osteoblast proliferation on the 50/50 nanophase alumina/PLLA composites was significantly ($p < 0.05$) higher for all durations (3, 7, and 14 days) tested (Figure 3). In addition, osteoblast proliferation was significantly ($p < 0.05$) higher on the nanophase than on the micron-size 50/50 alumina/PLLA composites after 3 and 7 days (Figure 3). Furthermore, osteoblast proliferation increased significantly ($p < 0.05$) with nanophase alumina loading (Figure 4).

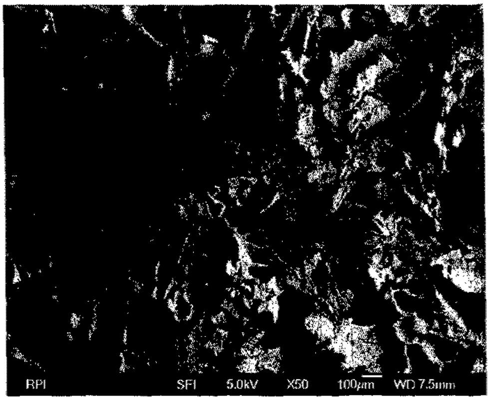

Figure 1. Representative scanning electron micrograph illustrating the porous architecture of the scaffolds tested in the present study. This picture is of a 50/50 nanophase alumina/PLLA composite. Bar = 100μm.

Table I
Pore-size and Open-porosity of PLLA and Alumina/PLLA Composites

	Pore-size (μm)	Porosity (%)
PLLA	145 ± 48	93.6 ± 0.3
50/50 micron-size alumina/PLLA	183 ± 68	91.9 ± 0.3
50/50 nanophase alumina/PLLA	152 ± 48	92.2 ± 0.3

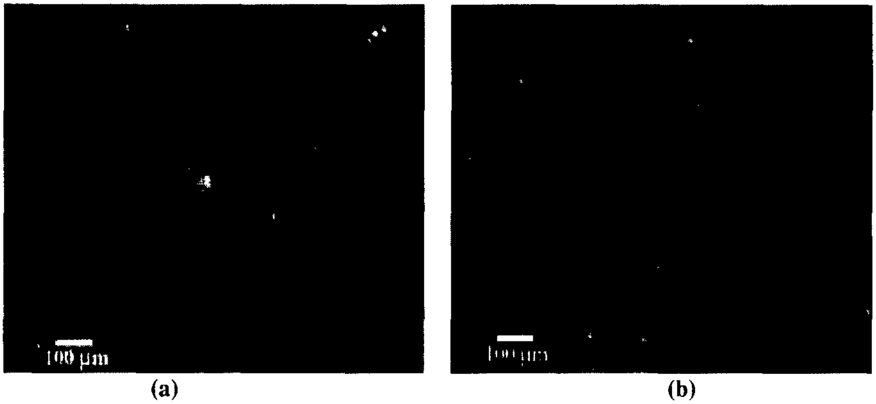

Figure 2. Representative fluorescence micrographs illustrating the osteoblast (light spots) distribution on a 50/50 nanophase alumina/PLLA composite 16 hours after seeding: (a) scaffold surface (0.5 mm from the scaffold edge); (b) scaffold center (5.0 mm from the scaffold edge). Bar = 100 μm.

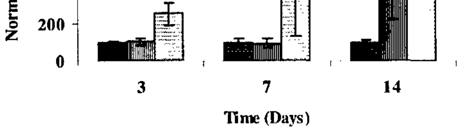

Figure 3. Time course of osteoblast proliferation on ■ pure PLLA (controls), ■ 50/50 micron-size alumina/PLLA, and ▤ 50/50 nanophase alumina/PLLA. The data were normalized against the cell proliferation results obtained on pure PLLA at the respective times. Values are mean ± SEM; n = 3; *$p < 0.05$ and $^{\#}p < 0.05$ compared to PLLA and micron-size composites, respectively (two-sample t test).

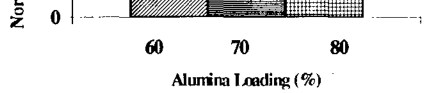

Figure 4. Osteoblast proliferation on ▨ 60/40 nanophase alumina/PLLA, ■ 70/30 nanophase alumina/PLLA, and ▤ 80/20 nanophase alumina/PLLA composites after 7 days of culture. The data were normalized against the cell proliferation results obtained on pure PLLA at day 7. Values are mean ± SEM; n = 3; *$p < 0.05$ compared to 60/40 nanophase alumina composites (two-sample t test).

Discussion

The present study was successful in preparing three-dimensional nanophase and micron-size alumina/PLLA composite scaffolds with pore-size, porosity, and compressive mechanical properties comparable to those reported in the literature for pure PLLA and micron-size hydroxyapatite/PLLA composites prepared using the same thermal phase separation technique.[7] Additionally, the absence of detectable degradation for all scaffold types is in agreement with literature reports of no significant weight loss for porous PLLA scaffolds under conditions similar to those used for the present study for durations of less than 50 days.[10]

In contrast to the differences observed in bending modulus for two-dimensional, non-porous nanophase and micron-size composites of alumina with either PLLA or poly(methyl-methacrylate),[2] the mechanical properties of all three-dimensional scaffolds examined by the present study were similar. An investigation of the underlying mechanisms for this discrepancy was outside the scope of the present study, but a possible explanation for these results is that the porous structure of the scaffolds masks the advantages provided by the nanophase materials tested.

Necrosis of cells in the center of three-dimensional scaffolds has been a problem encountered by investigators working in tissue engineering. For example, Ishaug et al. (1997) reported that cells further than 240 μm from the surface of poly(D,L-lactic-co-glycolic acid) scaffolds (disks 7 mm in diameter and 1.9 mm in height) did not survive during 56 days of culture.[11] Ma et al. (2000) reported cell necrosis in the center of pure PLLA scaffolds, but not in the center of micron-size hydroxyapatite/PLLA scaffolds (disks 10 mm in diameter and 1.5 mm in height).[12] In the present study, while osteoblasts in the center of alumina/PLLA scaffolds ($10 \times 10 \times 3$ mm^3) did not survive, cells present in the outermost (approximately 500 μm from the nearest surface) region of each scaffold remained viable for all durations (up to 7 days) tested. The exact causes for these observations are not known. However, since the scaffold degradation results of the present study preclude decreases in the media pH (due to polymer degradation), the observed cell necrosis is most likely due to limited diffusion of oxygen and nutrients under the static cell-culture conditions utilized routinely for such studies; this explanation was proposed by Sikavitsas et al. who have attempted to engineer bone tissue using cultures of marrow stromal cells on three-dimensional, porous scaffolds of 75/25 (w/w) percent poly(D,L-lactic-co-glycolic acid).[13]

The increased proliferation of osteoblasts on three-dimensional, nanophase alumina/PLLA composites reported in the present study is in agreement with a similar cell proliferation trend observed on essentially two-dimensional substrates of nanophase alumina/PLLA composites containing 30 - 50% alumina.[2] Important contributions of the present study are, therefore, the successful preparation of composites with nanophase alumina content as high as 80%, and the evidence that these formulations maintain the select and enhanced cytocompatibility first observed on essentially two-dimensional nanoceramics and on their composites with polymers.[2-4] The mechanism(s) behind the observed increased osteoblast proliferation as a function of alumina content is not known. Previous work in our laboratory, however, suggests that enhanced calcium adsorption may result in calcium-mediated binding and conformational changes of select proteins on both nanoceramic and nanoceramic/polymer composite surfaces; since proteins mediate cell interactions on substrates, these events play a key role in the subsequent cell adhesion, as well as in other osteoblast functions that are pertinent to new bone formation.[5]

Overall, the present study is the first to investigate some aspects of the use of three-dimensional, porous, nanophase alumina/PLLA scaffolds, which are pertinent to tissue engineering and implantable biomaterials. Undoubtedly, further research is needed to determine the optimal conditions under which these novel material formulations perform best in biomedical applications.

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