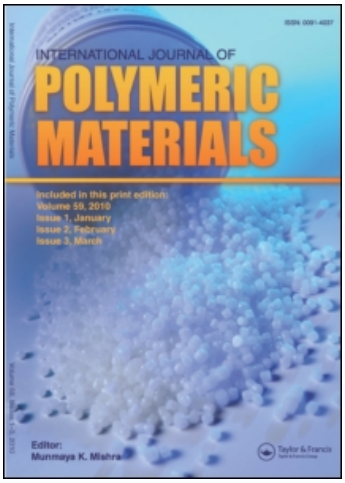

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Cytocompatibility of Poly(L-lactide-co-glycolide) Porous Scaffold Materials for Tissue Engineering

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The development of a suitable three-dimensional scaffold for the maintenance of cellular viability and differentiation is critical for applications in periodontal tissue engineering. Poly(L-lactide-co-glycolide) (PLGA) porous scaffold materials were fabricated by using $NaHCO_3$ particulates as progen, and the compatibility test in vitro was carried out. The scaffold shows an interconnected structure with large pore sizes ranging from 100 to 450 μm. Cells did attach to the culture plate when cultured in PLGA extract, and the number of cells increased with the increase of culture time. Von Kossa-positive mineralization nodules were first observed after 21 days of culture time. The percent of viable cells cultured in PLGA extract was higher than that in PLLA extract at the same culture period. Nodule formation and multilayer structures were observed on the scaffold surface.

Keywords: cytocompatibility, poly(L-lactide-co-glycolide), scaffold materials

INTRODUCTION

Biodegradable polymers are used in an increasingly large number of biomedical applications such as drug-releasing implants [1], bioresorbable surgical sutures [2], short-term fixation devices in the orthopedic field [3] and tissue engineering [4,5]. The most important characteristic of a biodegradable material relates to the biocompatibility of both the polymer (at implantation time) and the products of its degradation process.

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Tissue engineering using cell transplantation appears to be the most promising alternative to existing therapies for restoring tissue and organ function [6–8]. Significant challenges to this technique include the design and fabrication of a suitable biodegradable cell scaffold that can promote cell adhesion, support cell growth, proliferation and differentiation, and guide the process of tissue development [8–10]. Transport issues are very important for tissue engineering scaffolds and include nutrients delivery, waste removal, exclusion of materials or cells, protein transport, and cell migration, which, in turn, are governed by the scaffold's porous structure [11].

Biodegradable polymers, such as poly-L-lactic acid (PLLA), often are utilized to construct cell scaffolds for tissue engineering use. However, the hydrophobicity of the scaffolds inhibits the cells from penetrating into the pores. In addition, there are no cell recognition sites on the surfaces of this kind of polylactide scaffolds which affects their cell affinity. Many efforts have been directed towards enhancing cell affinity of scaffold materials. For example, copolymers have been widely investigated to improve the hydrophilicity of polymeric materials [12,13]. Poly(L-lactide-co-glycolide) (PLGA) is presently utilized in different forms to repair and regenerate the impaired tissues due to its faster degradation speed and higher hydrophilicity than PLLA. Its degradation products are low molecular mass compounds such as lactic acid and glycolic acid, which enter into normal metabolic pathways.

The purpose of the present study is to fabricate three-dimensional PLGA (85/15) scaffold materials with interconnected pore structure. Human osteoblastic cells were used to evaluate the biocompatibility in vitro of the scaffolds.

EXPERIMENTAL

Fabrication of Scaffolds of Biodegradable Polymer

PLGA (85/15) ($M_w = 9.4 \times 10^4$, $M_w/M_n = 1.55$) was obtained under high vacuum in the presence of stannous octoate as the catalyst at 140°C for 24 h. The PLGA was purified by dissolution of the polymer in chloroform and precipitation in ethanol, followed by drying in vacuum at room temperature for 48 h.

PLGA scaffolds were fabricated by using an improved solution-casting/salt-leaching technique. Briefly, PLGA was first dissolved in chloroform and sieved $NaHCO_3$ particulates (the diameter 300–450 μm) were added. The mixture was cast into a mold (Ø8.5 × 5 mm). After the evaporation of the solvent, the samples were dried for 24 h at room temperature (25°C) in air, then were placed in vacuum for

another 24 h at 40°C in order to completely remove the solvent. The samples were immersed in 12 wt% hydrochloric acid for 3 h, then washed 3 times with distilled water, then immersed in distilled water for 12 h, and the distilled water was refreshed every 4 h, all at room temperature. The samples were dried for 24 h in vacuum at 40°C. The polymer scaffolds were obtained after drying. The porous structure of the PLGA scaffold was characterized by scanning electron microscope (KYKY-2800) after being sputter-coated with gold.

Cell Morphology Cultured in Extracts

Human osteoblastic cells were obtained from the marrow of a young adult male. The extract medium for cells was Dulbecco's modification of Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 units $\cdot$ mL^{-1} penicillin and 50 μg $\cdot$ mL^{-1} streptomycin. The ratio of the volume of the extractant to the surface area of the material was 1 mL $\cdot$ cm^{-2}. The extraction process was carried out in a water bath at 37°C in 75 cm^3 flasks which were shaken at a speed of 60–65 rpm. After extracting for 48 h, the extracts were passed through a 0.22 μm filter, then stored at −20°C. Cells were seeded in 96-well plate with cell seeding densities of 1×10^5 in a humidified incubator containing 5% CO_2 at 37°C. On the second day of the experiment, the medium from each well was removed and replaced with 2 mL of extraction medium. Cell morphology was observed under a reverse microscope (TE2000U, NIKON).

Von Kossa Staining

For the bone nodule formation assay, mineralized matrix was evaluated by Von Kossa staining. Cell cultures were rinsed with phosphate-buffered saline solution (PBS) and fixed in 4% formaldehyde in PBS for 10 min. After washing with distilled water, cells were treated with 5% silver nitrate solution and kept for 30 min in a dark room. The excess silver nitrate solution was then completely washed away using distilled water, then the culture plate was treated with sodium carbonate/formaldehyde solution for a few minutes to develop color. Residual silver nitrate was neutralized with 5% sodium thiosulfate.

MTT Assay

The procedure followed for this test was as described previously and corresponds to the ISO MEM elution test for short-term cytotoxicity assessment [14]. Briefly, PLGA and PLLA samples were placed in

conic tubes with Dulbecco's modified Eagle medium and placed under constant shaking (60–65 rpm) at 37°C for 24 h for the materials to release eventual leachables (formation of extracts from the materials). After 24 h, the extracts were filtered through a 0.22 μm pore size filter, placed in contact with an 80–90% confluence cells' monolayer and further incubated for 72 h. After this time period, the viability of the cells was evaluated by the MTT test by measuring the optical density at 570 nm. All the materials were tested in 10 replicates for each extract for at least two independent experiments with reproducible results. The results are expressed as the absorbance of optical density as mean ± standard errors.

Cell Morphology on Surface of PLGA

Cell attachment and cell morphology on PLGA surface were studied. Sterilized scaffold was cut into $5 \times 5 \times 1$ mm pieces and washed in phosphate-buffered saline (PBS) twice, each for 5 min, then transferred into 24-well polystyrene culture plates using 2% agar (to prevent floating). 3 mL medium were added to the wells to prevent the cover slide from floating during cell seeding. Into each well, 1.0×10^5 cells (in 1 mL of medium) were then placed, and the plates were incubated at 37°C and 5% CO_2 for 7 days. The scaffolds were left undisturbed in an incubator for 45 min to allow the cells to attach to the scaffolds, after which time an additional 2 mL culture medium containing 20 μL dexamethasone was added to each well. Medium was changed every two days.

The cell morphologies on PLGA scaffold surface were studied by scanning electron microscope (KYKY-2800). The scaffolds cultured with cells for 21 days were fixed with 2.5% glutaraldehyde for 3 h at room temperature, then rinsed three times with PBS, and dehydrated in a grade ethanol series. Samples were then critical-point dried, coated with gold, and observed by SEM.

RESULTS AND DISCUSSION

Morphologies of PLGA Porous Scaffolds

SEM photomicrographs of cross-sections of scaffolds are shown in Figure 1. It can be seen that the porous structure of the scaffold is interconnected and the size range of the large pores is 100–450 μm when using $NaHCO_3$ particulates (93.7 wt%) as porogen. The porous materials with high porosity allow the migration or entry of cultured cells into the scaffold. The PLGA scaffolds have an enormous specific

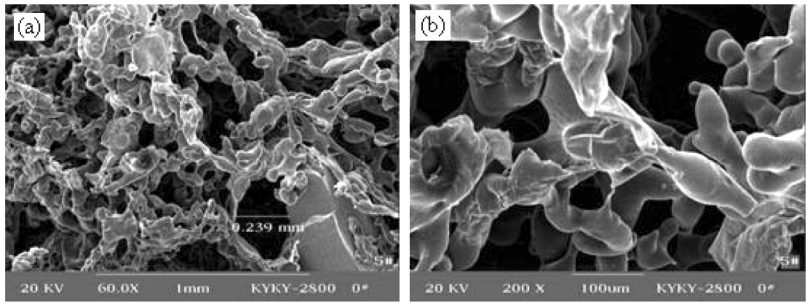

FIGURE 1 SEM micrographs of cross-section of PLGA scaffolds: (a) Low magnification; (b) High magnification.

surface area for cells to seed and interact with the materials, and the porous structure allows nutrients to diffuse into the scaffold to support the growth on the seeded cells.

An ideal scaffold for bone tissue engineering applications should be biocompatible (not toxic) and act as a three-dimensional (3D) template for in vitro and in vivo bone growth. It therefore must consist of an interconnected macroporous network with a modal interconnected pore diameter of at least 100 μm to allow cell migration, bone ingrowth and eventually vascularization [15]. The scaffold material should be one that promotes cell adhesion and activity and ideally stimulates osteogenesis at the genetic level [16] so that a tissue-engineered construct can be grown in vitro, ready for implantation.

Cell adhesion is an important factor of concern when biodegradable polymeric material is used as cell scaffold in tissue engineering. Hydrophilicity/hydrophobicity, surface energy, charge and roughness of the material surface greatly influence the cell attachment and cell growth on the material.

Morphology of Human Osteoblastic Cells Cultured in Extracts

Cells appeared to show normal morphology, with cell sizes ranging from 10 to 20 μm in diameter, when osteoblasts were cultured in PLGA extract (seen in Figure 2). Human osteoblastic cells were attached to the culture plate, which indicated that cells had strong generation abilities. It is apparent that the number of the cells increased in PLGA extract with increasing culture time.

In tissue engineering, it is crucial for cells to easily spread, migrate, proliferate, and secrete extracellular matrix (ECM) in 3D scaffold. It is known that cell shape is important for the growth,

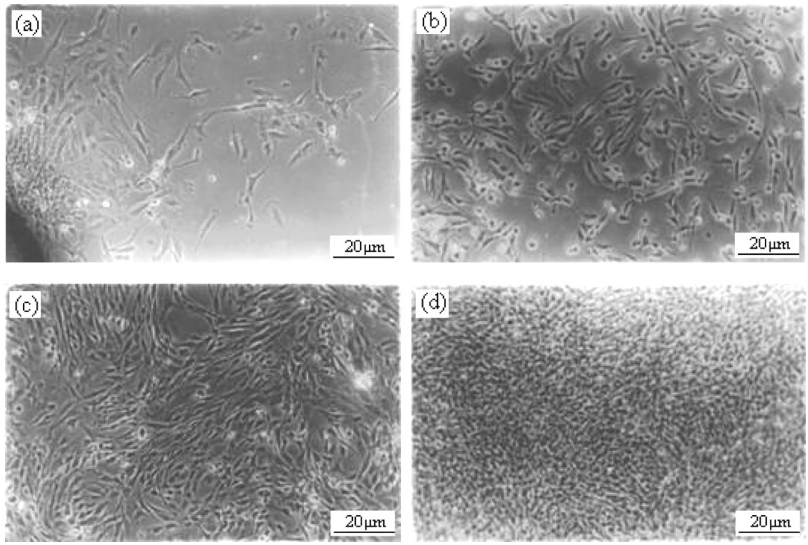

FIGURE 2 Morphology of cultured human osteoblastic cells in extracts of PLGA: (a) 1d; (b) 3d; (c) 1w; (d) 3w.

function, and even survival of the anchor-dependent cells [17–19]. The signals originated by adhesion plaque activation and mediated by integrins and cytoskeleton can be processed by the nuclear matrix and are essential for the regulation of the gene expression [20,21]. Chen et al. [17] proposed that cells with a flat shape survive better and proliferate faster than cells with a more rounded shape. Therefore, one can conclude that the spreading shape and the even distribution of the human osteoblastic cells in the PLGA scaffolds imply an obviously good cytocompatibility.

Mineralized Matrix Formation During Osteoblastic Differentiation of Human Osteoblasts

Mineralized nodule formation is basically considered to be the final result of differentiation and function of the osteoblasts. We used Von Kossa staining, a technique commonly used to detect calcium deposition in osteoblast cultures, for assessment of mineralized matrix formation in human osteoblastic cells.

Mineralized nodule of the newly formed bone is markedly positive (brownish-yellow precipitates) for Von Kossa staining. In our study, Von Kossa-positive mineralization nodules were first observed at day 21 followed by an increased number of positive nodules during

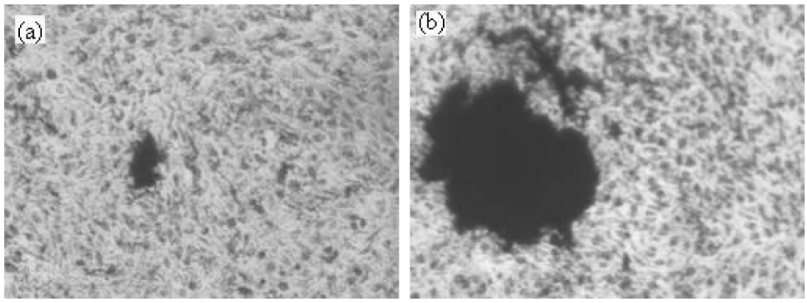

FIGURE 3 Von Kossa staining of human osteoblastic cells culture in intro: (a) 3 w; (b) 5 w.

the remaining culture period (see Figure 3). Production of mineralized nodules is one of the most reliable variables for evaluating the osteoblastic differentiation of human osteoblastic cells. These results suggest the occurrence of osteoblastic differentiation and mineralization of human osteoblastic cells cultured in PLGA extract.

Cell Proliferation and Viability

The MTT test is a biochemical test widely used to assess cytotoxicity by measuring cell viability and proliferation in a qualitative way. This biochemical test is based on the reduction of MTT (which is water-soluble and has a yellow tonality) by the cell mitochondrial enzyme succinate dehydrogenase, yielding a purple-colored salt insoluble in water. The salt absorbs light at a wavelength of 570 nm and because only living cells have the capability to metabolize the MTT, it gives a measurement of the viable cells.

The absorbance values of human osteoblastic cells cultured in extracts are shown in Figure 4. It indicates that the percent of viable cells cultured in PLGA extract was slightly higher than that in PLLA extract during the culture period. However, there is no significant difference ($P > 0.05$), which demonstrates that the cell affinity of PLGA scaffold is only slightly better than that of PLLA material.

Cell Attachment and Morphology on the Surface of PLGA

PLGA scaffolds were immersed in human osteoblastic cells culture. After three weeks of cell culture, the SEM picture (Figure 5) confirmed the presence of cells in confluent (multi-) layers on the surface of the scaffold. These cells had spread extensively and exhibited filopodia.

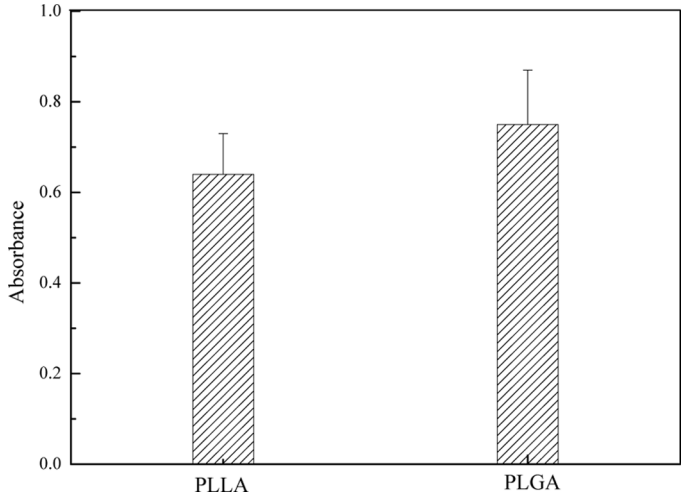

FIGURE 4 MTT test on cell cultures after 3 weeks from osteoblast plating.

The cells migrated into the pores and adhered to the walls of the pores through the filopodial extensions. Cell metabolism was extremely vigorous and a great deal of extracellular matrix (ECM) was secreted. The presence of a collagen ECM shows that the osteoblastic cells are capable of forming a matrix suitable for mineralization, and may indicate initiation along a biomineralization pathway.

Cells, scaffolds, and growth factors are the three main factors for creating a tissue-engineered construct. The development of good

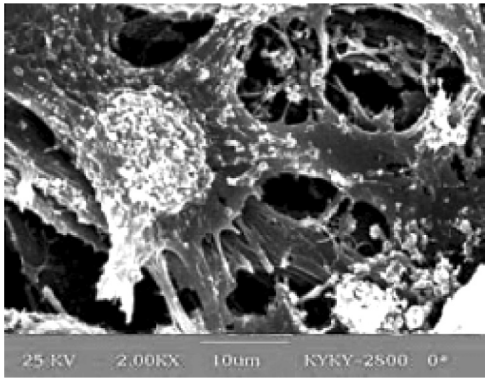

FIGURE 5 SEM morphology of human osteoblasts cultured on PLGA scaffold surface after 3 weeks.

biodegradable polymers to perform the role of a temporary matrix is an important factor in the success of cell transplantation therapy. The objective of this study was to evaluate PLGA materials as prospective candidates for periodontal tissue engineering. The optimal scaffold should support the movement, proliferation and differentiation of specific cells. In the present study, the porous PLGA scaffold exhibited a well-developed pore structure. The PLGA scaffold appeared to provide a good environment for the growth of human osteoblastic cells. After 3 weeks incubation, cells adhered, spread, and formed a monolayer on the surfaces of the scaffold. The human osteoblastic cells produced extracellular matrices to fill the voids in the scaffold.

CONCLUSIONS

Highly porous PLGA scaffolds with interconnected pores were obtained with $NaHCO_3$ as porogen. PLGA scaffold supports the attachment, proliferation and mineralized nodule formation of human osteoblasts. It provides an appropriate environment for the proliferation and differentiation of osteoblasts. During short culture term in PLGA extract, cells displayed normal morphology features, and Von Kossa-positive mineralization nodules could be observed after 3 weeks. Multilayer structures and nodules were formed on the scaffold surface after 21 days of culture time.

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