In Vivo Evaluation of Butylene Terephthalate-ethylene Oxide-DL, Lactide Polymer as Porous Scaffolds for Tissue Engineering

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ABSTRACT: The copolymers of poly(1,4-butylene terephthalate-co-ethylene oxide-co-DL-lactide), obtained by the transesterification reactions of poly(butylenes terephthalate) with poly(ethylene glycol DL-oligo(lactic acid), were fabricated into porous scaffolds by the established solvent-casting and particulate-leaching technique with NaCl as the porogen. The morphology of the porous scaffolds were investigated by the scanning electron microscopy (SEM), and the pores within the scaffold were proven to be interconnective ranging in size from 200 to 400 μm. The human bone marrow mesenchymal stem cells (MSC) seeded on the scaffolds were confirmed to survive and proliferate within the pores of the scaffold with the observation by immunofluorescence microscope and SEM. *In vivo* implantation of MSC-seeded scaffolds into athymic nude mice showed significant tissue formation in the subcutaneous sites of the immunodeficient mice at 3, 4, 6, and 9 weeks. The results indicate that the scaffolds were biocompatible with MSC and the host tissue *in vitro* and *in vivo*.

KEY WORDS: poly(1,4-butylene terephthalate-co-ethylene oxide-co-DL-lactide), scaffold, tissue engineering.

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INTRODUCTION

There is an interest in developing biodegradable scaffolds for use in biomedical applications [1,2]. The biomaterials used for the preparation of scaffolds need to have adequate mechanical strength and flexibility as well as to be able to support the regenerating cells and tissue. Synthetic polymers such as poly(lactic acid) (PLLA), [3–6] poly(lactic acid-co-glycolic acid) (PLGA) [7–14] and poly(lactic acid-co-caprolactone) [15–18] have been extensively used in biomedical applications because of their availability, ease of processing, low inflammatory response and approval by the FDA. Additionally, native biopolymers including collagen [19,20] and chitosan [21] have also been demonstrated to be effective in the biomedical applications.

For bone tissue engineering and vascular scaffolds applications the above polymers are either expensive or present physical and thermo-mechanical properties (such as, melting and glass transition temperature) which are significantly lower than those of conventional thermoplastics. Biocompatible copolyesters possess fast biodegradation and good biocompatibility due to the low aromatic sequence length and incorporation of aliphatic sequence in the copolyesters. Reactive blending of already existing homopolymers has been proven to be a successful and inexpensive way to produce new alphatic/aromatic copolyesters with intermediate properties [22–26]. In our previous work, the copolymer of poly(1,4-butylene terephthalate-co-ethylene oxide-co-DL-lactide) (PBTEOLA) was synthesized by the transesterification reactions of poly(butylenes terephthalate) (PBT) and poly(ethylene glycol)/DL-oligo(lactic acid) (PEG/OLA) [27]. The copolymer was found to show enhanced mechanical performance and sufficient biodegradability [27].

For tissue engineering, the biomaterials are always fabricated as 3D porous scaffolds. There are several preparation methods being used, including porogen leaching [28], thermally induced phase separation [29], freeze-extraction, and freeze-gelation methods [30,31]. Solvent-casting and particulate-leaching with sodium chloride (NaCl) as the porogen is considered to be a simple and effective way [32].

The human bone marrow mesenchymal stem cells (MSC) are pluripotent progenitor cells that can form a variety of cells, including fat cells, cartilage, bone, tendon, and ligaments, muscles cells, skin cells, and nerve cells. MSC represent an ideal stem cell source for cell therapy; it is easily isolated from bone marrow, purification and amplification and multipotency. MSC have the ability to proliferate *in vitro*, retaining their adipogenic, chondrogenic, and osteogenic potential, which is

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reflected by the simultaneous expression of gene characteristics of various mesenchymal cell lineages. The advantages that MSC have makes them good candidates for use in tissue repair and other clinical applications, including bone regeneration [33–36].

The copolymers of PBTEOLA, with good mechanical and biodegradable properties, was successfully synthesized. The PBTEOLA copolymers exhibited a Young's modulus of 96 MPa and were degraded completely within 11 months *in vivo*. In this article, the PBTEOLA copolymers porous scaffolds aimed at bone tissue engineering applications were fabricated by the solvent-casting and particulate-leaching technique. The porous scaffolds were seeded with MSC cells and implanted in the subcutem of rats. The morphology of the scaffolds were observed by the scanning electron microscopy (SEM) and the MSC seeded on the scaffold were examined by the immunofluorescence microscopy. The *in vivo* experiments confirmed that the scaffold showed good compatibility to both the cells and the host tissue.

EXPERIMENTAL SECTION

Preparation of PBTEOLA Scaffolds

The copolymer of PBTEOLA was synthesized as described previously [27]. The reactive blend of 70% of poly(1,4-butylene terephthalate) (PBT), 25% of poly(ethylene oxide) (PEG) and 5% of oligo(lactic acid) (OLA) in weight percentage gave PBTEOLA copolyester containing butylenes terephthalate (BT), ethylene oxide (EO), and lactide (LA) units in molar ratio of 36: 59: 5. The M_w and M_n of the copolymer are 6.5×10^4 and 4.2×10^4 g/mol, respectively, as determined by gel permeation chromatography (GPC) with a Breeze Waters system on Styragel HT3 and HT4 columns using chloroform as eluent and monodisperse polystyrene as the calibration standards. pPly(ethylene glycol), $M_n = 1000$ g/mol, and α-hydroxy-ω-carboxyoligo(lactic acid) (OLA), $M_n = 1700$ g/mol, were used. The sieved NaCl particles ($200 \leq$ particle size $\leq 400\,\mu$m) and chloroform was purchased from the Damao Chemical Regent Factory of Tianjin.

The PBTEOLA was processed into porous foams by solvent-casting, particulate-leaching technique with NaCl as the porogen [32]. Briefly, NaCl particles (100–200 μm) 10 g were dispersed in a solution of 0.5 g PBTEOLA in 5 mL of choloform. The dispersion was then cast in a crucible (2 cm in diameter); the samples obtained had a diameter of ~2 cm and ~4 mm thick. These were air-dried for 24 h and then vacuum-dried at 100 μm Hg for 24 h to remove the remaining solvent.

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The resulting PBTEOLA/salt composite was immersed into 1000 mL of distilled deionized water for 24 h (the water changed every 6 h). After leaching out the salt, the product was air and vacuum-dried. For *in vitro* and *in vivo* tests, the scaffolds were cut into $0.5 \times 0.5 \times 0.2$ cm pieces.

SEM Observations

The morphology of the scaffold was studied by scanning electron microscopy (SEM), using a Hitachi S-570 electron microscope. The scaffold was mounted on the sample studs by means of double-sided adhesive tape. A thin layer of platinum was sputtered on the sample surface prior to SEM measurements at an accelerating voltage of 5 kV. For cross-sectional view studies, the membrane was fractured under liquid nitrogen prior to the SEM measurement.

Cell Seeding and Transplantation

The human bone MSC were supplied by the Institute of Urology Surgery of The First Affiliated Hospital of Nanchang University. The MSC were cultured in α-MEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (National HyClone (Lanzhou) Bio-Engineering Co., China), 100 U/mL penicillin (Sigma, St. Louis, MO), 100 μg/mL streptomycin (Sigma) and 25 μg/mL DAPI (Sigma). Cells were incubated at 37°C in a 5% CO_2 incubator and the medium was changed every 2 days. After the cells reached confluence, they were harvested by trypsinization (0.25% trypsin and 0.04 mol EDTA (disodiumethylenediaminetetraacetic acid) in PBS (phosphate buffer solution)) followed by the addition of fresh culture medium to create a cell suspension. After washing by PBS and centrifugation, a highly concentrated suspension (5×10^6 cells/mL) of MSC was obtained.

Before cell seeding, the scaffolds were sterilized with 75 vol% ethanol (Damao Chemical Regent Factory of Tianjin) for 1 h and then incubated in PBS for 24 h to exchange ethanol. The MSC suspensions (5×10^6 cells/mL) were dispersed into the scaffolds by injection using a syringe. The cell-containing scaffolds were then incubated at 37°C in a 5% CO_2 incubator for 4 h. The cells seeded in all the specimens were obtained from the same donor.

Morphology of the MSC on the Scaffolds

After culturing for 5 days, the scaffolds with MSC were stochastically isolated and observed under the immunofluorescence microscope.

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The karyons exhibiting a blue fluorescence were recognized to be the MSC tagged by DAPI.

After culturing for 5 days, the MSC grown on different scaffolds were washed with PBS to remove nonadherent cells, fixed in 4% glutaraldehyde (Damao Chemical Regent Factory of Tianjin) for 1 h at room temperature, dehydrated through a series of graded alcohol series and then critical-point dried with hexamethyldisilazan (Aldrich) overnight to maintain the normal cell morphology. The dried cellular constructs were gold sputtered and observed under the SEM at an accelerating Voltage of 15 kV.

In Vivo Histological Investigation

The athymic nude mice were purchased from Shanghai China for Animal Experiments. The athymic male mice were obtained at 5 weeks and acclimated for 1 week before use. Under anesthesia by ether vapor, two transverse incisions were made on the dorsum of each mouse to create a subcutaneous pocket using a sterile surgical technique. Each animal received tow cell/scaffold constructs on the back. The cell-material constructs were harvested after *in vivo* cultivation for 2–9 weeks. Samples for histological evaluation were fixed in neutral buffered formalin (Damao Chemical Regent Factory of Tianjin), embedded in paraffin and sectioned in cross-section through the center of the implant (5 μm thick sections). The cross-sections were routinely stained with hematoxylin and eosin (HE).

RESULTS AND DISCUSSION

A new biodegradable copolyester PBTEOLA was prepared by a bulk melting reaction between a reactive blend of poly(ethylene glycol)/DL-oligo(lactic acid) (PEG/OLA) and poly(1,4-butylene terephthalate) (PBT). The existence of new heterolinkages inside the backbones of the copolyesters, was confirmed by H^1NMR [27]. When transesterification was performed at 260°C for 1.5 h, the copolymers obtained had nearly a statistical microstructure based on sequence and thermal analyses. During the transesterification lactide and ethylene oxide units were built into the polymer chain yielding poly(1,4-butylene terephthalate-co-ethylene oxide-co-DL-lactide) (PBTEOLA). The lactide had two different functional groups and the ethylene glycol had two hydroxyl end groups, so the transesterification between butylene terephthalate (BT), ethylene oxide (EO), and lactide (LA) units (or segments) proceeded as shown in Scheme 1. The copolyester underwent significant

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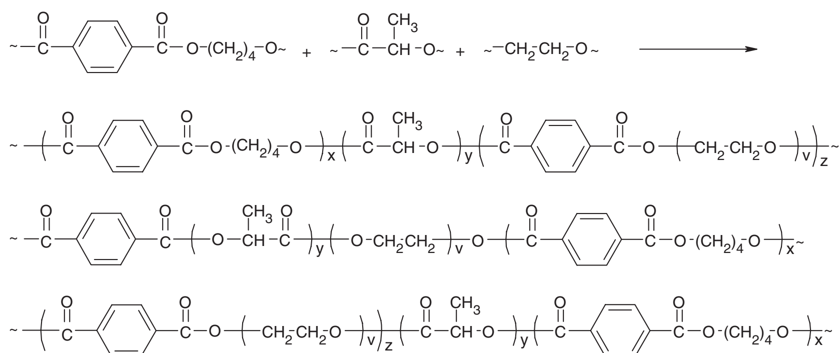

Scheme 1. Possible chemical structure in transesterification between PBT and PEG/OLA.

hydrolytic degradation in phosphate buffer solution (PBS) at 37°C, which increased with increased lactide and ethylene oxide composition. Hydrolysis occured mainly on the aliphatic ester groups, especially on the lactide ester bonds. The copolyester had a 10% weight loss after 4 weeks in a PBS (Fluka, pH 7.413) at 37°C.

The porosity and pore size of the scaffold are important features, sufficient pore size allows penetration and nutrient transfer for optimal tissue growth, however, there are conflicting reports on pore size with respect to new bone formation and growth [37–39]. The porosity and pore size can be controlled by the salt weight and particulate size. Shown in Figure 1 is the interconnected pore structure of a PBTEOLA scaffold. The scaffolds resembled the macroscopic contours of the porogen assembly and had a pore size of 200–400 μm with good interconnectivity between pores. A large number of smaller pores of size 10–30 μm were also found in the skeletons, produced by the bubbles formed during the evaporation of the solvent.

Bone marrow MSC represent an excellent stem cell source and candidates for use in tissue repair and clinical applications that were used in the study [33–36]. The MSC distributions within the pores of the scaffolds are shown in Figure 2. The bright dots distributed regularly in the background, indicate that the cells attached were growing well in the scaffolds. The porous structure of the scaffold provides more space for cell association and makes the penetrating of the cells into the scaffold easier as well as benefit the nutrient transformation and proliferation of the cells. Although mild acidic hydroxyl acids and oligomers occurs with degradation *in vitro* [27], these influences might not be serious as diffusion from the inner part of the scaffold to the outside can occur through the pores.

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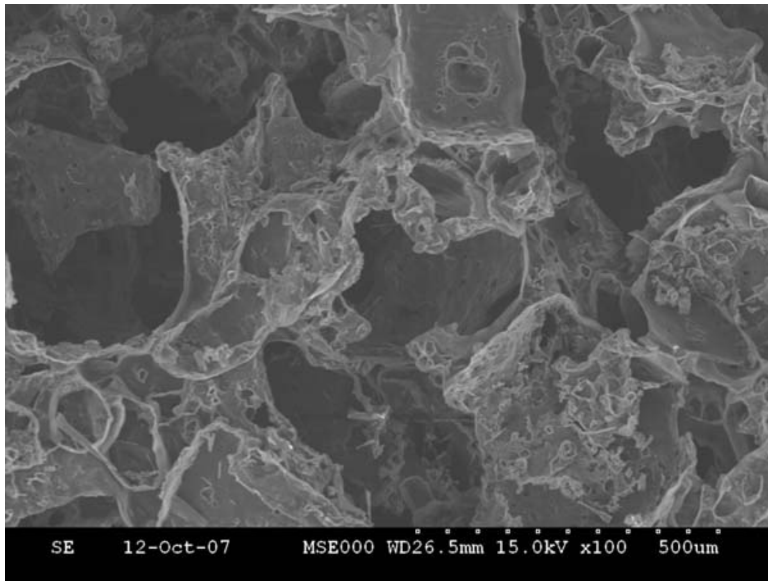

Figure 1. SEM images of porous PBTEOLA scaffolds.

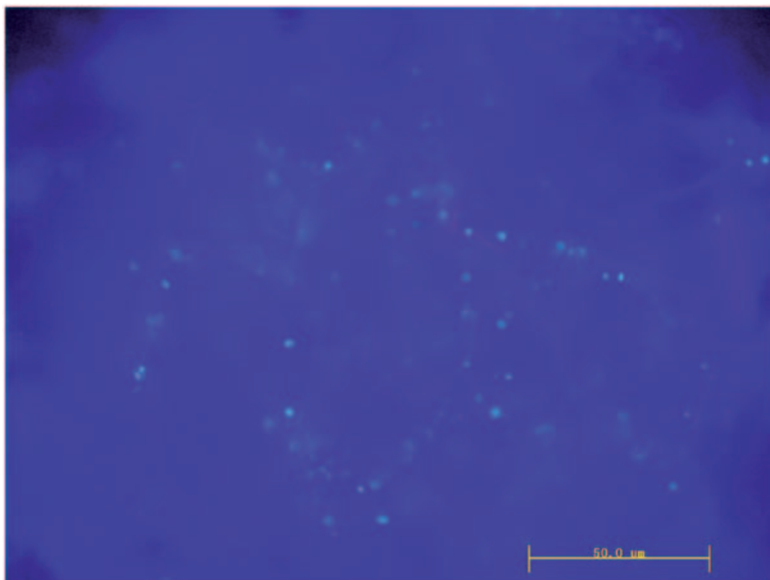

Figure 2. Immunofluorescence microscope images of the MSC seeded on the scaffold and cultured for 5 days.

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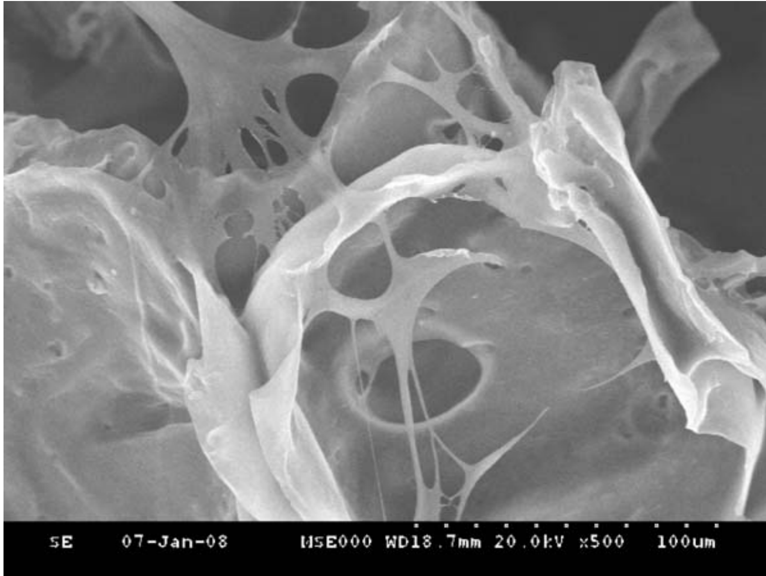

Figure 3. SEM images of the MSC seeded on the scaffolds and cultured for 5 days.

Cell morphology and cell-ECM interaction play a vital role in the strategy of tissue engineering. As shown in the SEM in Figure 3, the attachment of the MSC cultured on the scaffold have polygonal, elliposoidal, and other shapes with several short pseudopods as seen on the surface of the lumen within the scaffolds, indicating that the cells are surviving within the scaffolds. No evidence of toxicity or cell reactions to the scaffold was observed as there were no rounded cells, no inclusions or vacuoles in the cytoplasm, and the nuclei contained many well formed and defined nuclei. The cytoplasm also showed evidence of high synthetic activity showing rough endoplasmic reticulum. Preliminary investigations show a strong cell attachment and biocompatiblity to the copolyester scaffold.

The animals implanted with MSC containing PBTEOLA scaffolds survived for 9 weeks and further observations are being continued. The tissue integrated into part of the scaffold after 2 weeks and the tissue had integrated completely into the scaffold after 4 weeks. It was difficult to distinguish the tissue to the scaffold by naked eye. Histological sections are shown in Figure 4. The osteoid formation, osteocytes, and lacuna structures in newly generated tissue were observed. Although the PBTEOLA scaffold had not completely degraded during the 9-week implantation period (denoted by S in Figure 4),

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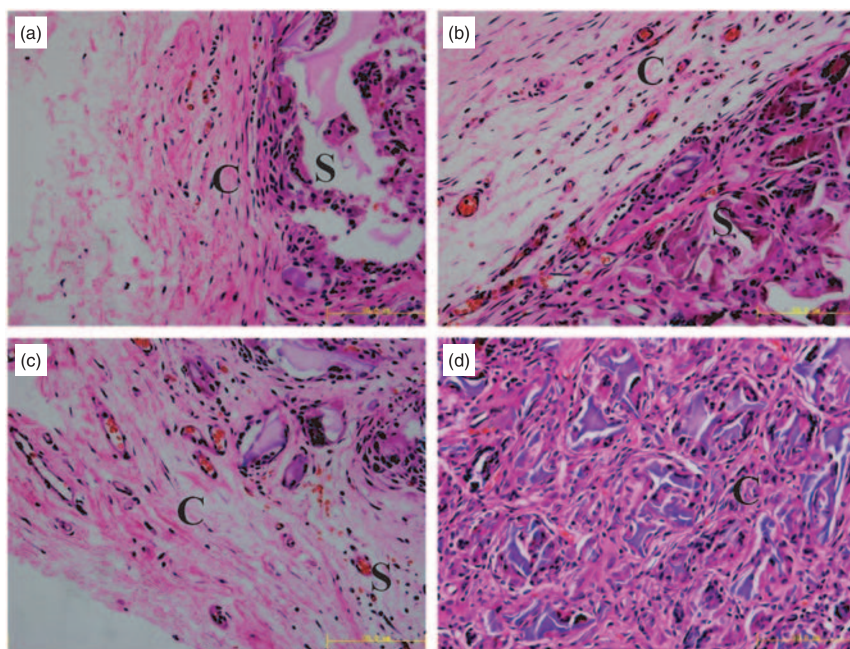

Figure 4. Histochemical evaluation of osteogenic induction of (a) 3 weeks, (b) 4 weeks, (c) 6 weeks, and (d) 9 weeks. All the samples were stained by hematoxylin and eosin. C and S in the Figures represent connective tissue and scaffold, respectively.

only fibrous connective tissue was observed in the new tissue of the scaffolds (denoted by C in Figure 4).

Depending on each time-point after implantation, there are noticeable changes in the areas occupied by scaffolds and newly formed tissues [13]. After 2 weeks, fibrocytes had regenerated around the scaffolds and little blood vessels had spread out. After 3 weeks, the amount of the fibrocytes decreased while the amount of the wavy fiber tissue increased (Figure 4(a)) and lymphocytes could be observed. After 4 weeks, the tissue around the scaffold became fibrous, little blood vessels outspreaded, and the number of lymphocytes decreased (Figure 4(b)). After 6 weeks, the tissue around the scaffold was completely fibrous and dispersed lymphocytes could be occasionally observed (Figure 4(c)).

After 9 weeks, the scaffolds were completely covered by new tissue and no inflammatory cells were discerned (Figure 4(d)). With time, the area occupied by the polymeric part of the scaffolds significantly decreased while the area occupied by the newly formed tissue increased. No obvious inflammatory response was observed after the scaffolds were

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implanted in the animals. The PBTEOLA scaffold had good biocompatibility with the host tissue and the degradation products did not affect the survival of the MSC implanted. However, at this time (9 months) the scaffolds were not completely degraded.

CONCLUSIONS

The PBTEOLA copolymer was fabricated to form porous scaffolds by the solvent-casting and particulate-leaching technique. SEM analysis confirmed that the pores within the scaffold were inter-connective with sizes 200–400 μm. After 5 days *in vitro* cell culture, the MSC were observed to distribute well in the scaffold pores as detected by the immunofluorescence microscope. SEM showed that the MSC had began to spread, presenting polygonal morphology with more pseudopods for 5 days. In vivo experiments indicated that the tissue penetrated into the scaffold in 4 weeks. The scaffold was biocompatible with both the cells and the host tissue. The degradation products produced by the scaffold showed no serious toxicity to the cells and the host tissue; no inflammatory responses were observed. This PBTEOLA copolymer has potential as a new class of biodegradable material for tissue engineering, such as bone regeneration.

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