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Special Theme Research Article

Manufacture of solvent-free polylactic-glycolic acid (PLGA) scaffolds for tissue engineering

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ABSTRACT: Conventional methods for fabricating polymeric scaffolds often use organic solvents which might be harmful to cells or tissues. The purpose of this report was to develop a solvent-free method for the fabrication of three-dimensional scaffolds for tissue engineering. To manufacture a scaffold, polylactide-polyglycolide (PLGA) copolymers were premixed with sodium chloride particulates. The mixture was then compression molded and sintered to form a cylinder. After sintering, the cylinder was submerged in water for 48 h to leach out the particulates. The scaffold, with approximately 2×10^7 mesenchymal stem cells (MSCs) of the New Zealand rabbit, was then cultured in an osteogenic culture medium for 14 days. The alkaline phosphatase activity, calcium level, and the mineral deposition of cultured cells in the PLGA scaffolds were determined. The results showed that an increase of alkaline phosphatase activity and calcium levels, as well as abundant mineral deposition, was observed in the cultured mesenchymal stem cells. In addition, scaffolds with pore sizes of 88–125 μm showed the most number of cells during the period of culture. Developing solvent-free biodegradable scaffolds for bone cells may provide a potential method for the treatment of infected bone defects. © 2008 Curtin University of Technology and John Wiley & Sons, Ltd.

KEYWORDS: biodegradable scaffold; polylactide-polyglycolide (PLGA); compression sintering; salt leaching; tissue engineering; mesenchymal stem cell

INTRODUCTION

Over the past decade, the main goal of tissue engineering[1] has been to develop biodegradable materials for the regeneration of many tissues and organs including bone,[2] cartilage,[3] liver,[4] skin,[5] peripheral nerves.[6] For the repair of bone defects, the ideal biomaterial is one that has mechanical properties similar to bone, can be fabricated easily into a desired shape, supports cell attachment, contains factors to induce the formation of new bone tissue, and biodegrades to permit natural bone formation and remodeling. In many tissue engineering applications, porous scaffolds with an open-pore structure are often desirable for maximizing production, vascularization, and tissue ingrowth. Research has focused on using biodegradable polymers as a scaffold to direct specific cell growth and differentiation.[7–9] Poly(α-hydroxy esters), such as poly(L-lactic acid) (PLLA) and poly(DL-lactic-co-glycolic acid) (PLGA), are among the few synthetic polymers approved for human clinical use.[10,11] They have been shown to be biocompatible, biodegradable, and easily processed. In addition, the physical, chemical, mechanical, and degradable properties of these materials can be engineered to fit a particular application.[12–16] These porous polymer scaffolds have been utilized as bone graft substitutes for filling large bone defects.

Most of the previous methods for fabricating polymeric scaffolds, such as the solvent-casting separation and particulate leaching method or the phase separation method, use organic solvents.[17–33] However, residual solvents in the scaffolds may be harmful to transplanted cells or host tissues.[34]

In this study, we developed a novel solvent-free method of manufacturing three-dimensional biodegradable scaffolds for bone tissue engineering by using compression sintering and salt leaching techniques. To manufacture a scaffold, polylactide-polyglycolide copolymers were premixed with sodium chloride particulates. The mixture was then compressed and sintered to form a cylinder. After sintering, the cylinder was submerged in water for 48 h to leach out the particulates. Scaffolds of three different pore sizes were thus obtained.

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The scaffold with approximately 2×10^7 mesenchymal stem cells (MSCs) of the New Zealand rabbit was then cultured in an osteogenic culture medium for 14 days. The alkaline phosphatase (ALP) activity, calcium level, and the mineral deposition of cultured cells in the PLGA scaffolds were determined. In addition, optical microscopy and scanning electron microscopy (SEM) were employed to observe the cell growth in the scaffolds.

MATERIALS AND METHOD

Materials

The polymers used were poly (DL)-lactide-co-glycolide with a ratio of 75 : 25 and an intrinsic viscosity of 0.8. All polymers were available in powder form with particle sizes ranging from 100 to 200 μm. A DuPont model TA-2000 differential scanning calorimeter was used to characterize the thermal properties of the polymer. The measured results suggested that the polymer's glass transition temperature was in the range of 50–60 °C. The sodium chloride used was of a commercially available grade. The sodium chloride was prepared by milling in an analytical mill and sieved to particles of three different size ranges: 50–88, 88–125, and 125–200 μm.

Fabrication of biodegradable scaffolds

To fabricate biodegradable scaffolds, the polymers and NaCl were first mixed by a lab-scale mixer. The salt/polymer mass ratio was 9 : 1. The mixture was then compression molded and sintered into a cylinder by a 304L stainless mold (Fig. 1). The thickness of the cylinders was 1.5 mm; the sintering temperature was set at 95 °C; and the sintering time was 120 min in order to attain an isothermal sintering[35] of the materials. After sintering, the cylinder was submerged in distilled water for 48 h to leach out the NaCl. During the leaching process, the cylinders swelled and open channels were formed as a result of the swelling. The sodium chloride was thus released through a channel diffusion mechanism. Figure 2 shows schematically the fabrication process of the scaffold. To ensure that the sodium chloride was completely released, the weight of the cylinder before and after the leaching process was measured. Table 1 lists the contents of the materials in the cylinders as well as the weight variations of the scaffolds by the leaching process. As can be seen from Table 1, all NaCl was leached out after the 48-h water submersion, which in turn minimized the potential damage of the sodium chloride to the subsequent cultured cells.

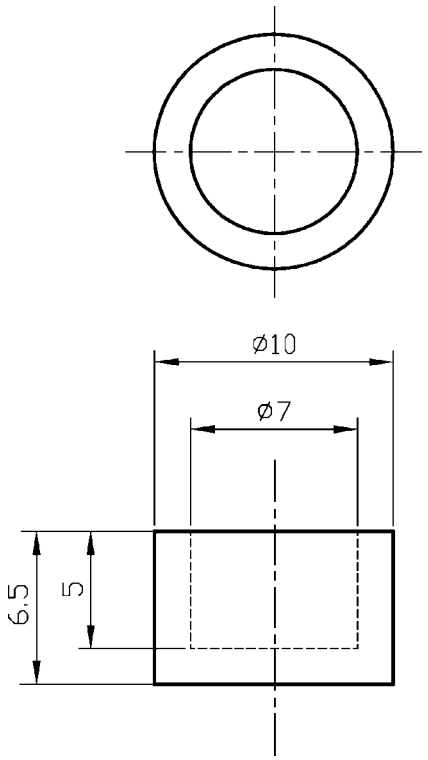

Figure 1. Dimensions of the biodegradable scaffolds.

Isolation and cultivation of rabbit MSCs

New Zealand rabbits weighing 3 kg were anesthetized by an intravenous injection of 5 ml of ketamine hydrochloride (Ketalar; Parke Davis, Taiwan) and Rompum (Bayer, Leverkusen, Germany) mixture. Under sterile conditions, 10 ml of bone marrow aspirated from the iliac crest was collected into a syringe containing 6000 units of heparin to prevent clotting. The marrow sample was washed with Dulbecco's phosphate-buffered saline (DPBS) and disaggregated by passing it gently through a 21-gauge intravenous catheter and syringe to create a single-cell suspension. Cells were recovered after centrifugation at 600 *g* for 10 min. Up to 2×10^8 nucleated cells in 5 ml of DPBS were loaded onto 25 ml of Percoll cushion (Pharmacia Biotech) of a density of 1.073 g/ml in a 50-ml conical tube. Cell separation was accomplished by centrifugation at 1100 *g* for 40 min at 20 °C. The nucleated cells were collected from the interface, diluted with two volumes of DPBS, and collected by centrifugation at 900 *g*. The cells were re-suspended, counted, and plated at 2×10^5 cells/cm^2 in T-75 flasks (Falcon). The cells were maintained in Dulbecco's Modified Eagle's Medium-low glucose (DMEM-LG; Gibco) containing 10% fetal bovine serum (FBS) and antibiotics (mixture of 100 units/ml of penicillin and 100 μg/ml of streptomycin; Gibco) at 37° in a humidified atmosphere of 5% CO_2 and 95% air. After 4 days of primary culture,

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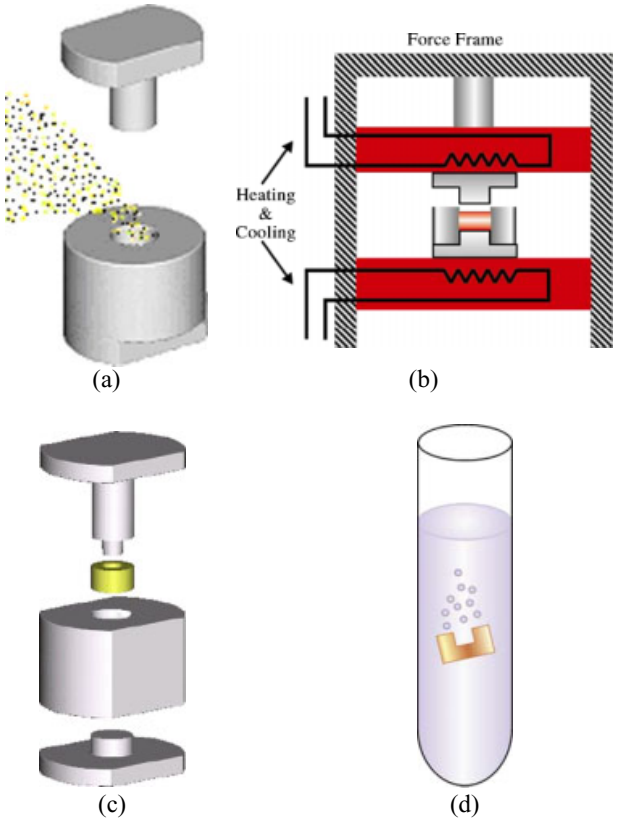

Figure 2. Schematically, the manufacturing process of the biodegradable scaffolds, (a) addition of PLGA/NaCl mixture into the mold, (b) compression sintering of the scaffolds, (c) demolding, and (d) salt leaching. This figure is available in colour online at www.apjChemEng.com.

the nonadherent cells were removed by changing the medium; medium was changed every 3 days thereafter. MSCs grew as symmetric colonies and were subcultured at 10–14 days by treatment with 0.05% trypsin and 0.53 mM EDTA for 5 min, rinsed from the substrate with serum-containing medium, collected by centrifugation at 800 *g* for 5 min, and seeded into fresh flasks at 5000–6000 cells/cm^2. Cultures were incubated in a humidified atmosphere of 5% CO_2/95% air until cell confluence. All animal procedures received institutional approval and all studied animals were cared for in accordance with the regulations of the National Institute of Health of the Republic of China (Taiwan), under the supervision of a licensed veterinarian.

DNA analysis

To determine the seeding efficiency and cell growth on the scaffolds, cell numbers were determined by quantitative DNA assays ($n = 3$). DNA was isolated using a Wizard Genomic DNA Purification kit (Promega, Madison, WI). For DNA isolation, the cell/scaffold constructs were washed twice with phosphate-buffered saline (PBS). The specimens were placed in a 1.5-ml tube and crushed with a homogenizer (PowerGen 125; Fisher Scientific, Germany). DNA was isolated according to the kit protocol, and DNA content was measured in triplicate on an ELISA plate-reader (MRX; Dynatech Labs).

Quantitative measurement of alkaline phosphatase activity

Since ALP is a cell-surface enzyme, ALP activity is measured in living cultures. The medium was withdrawn and the MSC carriers were washed twice with 10 ml of Tyrode's balanced salt solution. A 10-ml aliquot of ALP substrate buffer (50 mM glycine, 1 mM $MgCl_2$, pH 10.5), containing the soluble chromogenic ALP substrate (2.5 mM *p*-nitrophenyl phosphate), was added at room temperature. During incubation, cell-surface ALP converted *p*-nitrophenyl phosphate into *p*-nitrophenol that then took on a yellow color. Twenty minutes after substrate addition, 1 ml of the buffer was removed from the culture and mixed with 1 ml of 1 N NaOH to stop the reaction. The absorbance of the mixture was read in triplicate on an ELISA plate-reader (MRX; Dynatech Labs) at 405 nm and compared to serially diluted standards. Enzyme activity was expressed as *n* mole *p*-nitrophenol/min.

Calcium level quantification

Scaffolds with MSCs were rinsed twice with Tyrode's balanced salt solution, and then put in 50-ml tubes containing 10 ml of 0.5 N HCl. Calcium was extracted from the cells by shaking for 24 h at 4 °C. Cellular debris was centrifuged and calcium in the supernatant was measured quantitatively according to the manufacture's protocol in Sigma Kit #587. Absorbance of the

Table 1. Weight, size, and composition of the scaffolds.

Size of NaCl particles (μm)		50–88	88–125	125–200
Weights of scaffolds before water leaching	Polymer weights (mg)	41.7	55.2	56.9
	NaCl (mg)	379.4	497.6	500
Weights of scaffolds after leaching and drying		40.0	53.2	54.1

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samples was measured on the multiplate reader (MRX; Dynatech Labs) at 570 nm 5–10 min after the addition of pertinent reagents. Total calcium was calculated from standard curves of solutions prepared in parallel with the experiments and expressed as μg Ca/dish.

SEM observation

The surface morphology, scaffolding, and cell-scaffolding constructs were examined using a SEM. The samples were washed twice with PBS, prefixed in 1% (v/v) buffered glutaraldehyde for 1 h, and fixed in 0.1% (v/v) buffered formaldehyde for 24 h. The fixed samples were dehydrated in ascending grades of ethanol, dried and mounted on aluminum stubs using double-sided carbon tape. The specimens were coated with gold using a Sputter Coater and examined with SEM at an acceleration voltage of 10 kV.

Histological observation (Alizarin Red S Stain)

Fourteen days after culture treatments, MSC scaffold tissue samples were taken and fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were cut, deparaffinized, stained with Alizarin Red S (Sigma), and examined under the microscope. In the Alizarin staining, a 1-ml aliquot of freshly prepared 2% (w/v) Alizarin Red S (pH 4.2) in distilled water was added, and the sections were incubated for 3 min at room temperature. The presence of mineral deposition was indicated by the development of a red precipitate on the mineralized matrix.

RESULTS

Characterization of biodegradable PLGA scaffolds

Compression sintering and the subsequent salt leaching of scaffolds containing a high percentage (90%) of NaCl particles led to the formation of highly porous, open pore structures with no evidence of an external, nonporous skin layer (Fig. 3). Scaffolds of three different pore size ranges were fabricated: 50–88, 88–125, and 125–200 μm.

Osteoblast culture on PLGA scaffolds

The attachment and proliferation of human osteoblast-like cells on biomaterials is required for tissue engineering of bone autograft. The biodegradable scaffold fabricated in this study allowed for the adhesion and proliferation of the seeded osteoblasts over the 14-day *in vitro* culture period, as shown in Fig. 4. In addition, the scaffold with a pore size of 88–125 μm showed the most number of cell attachment during the period of culture.

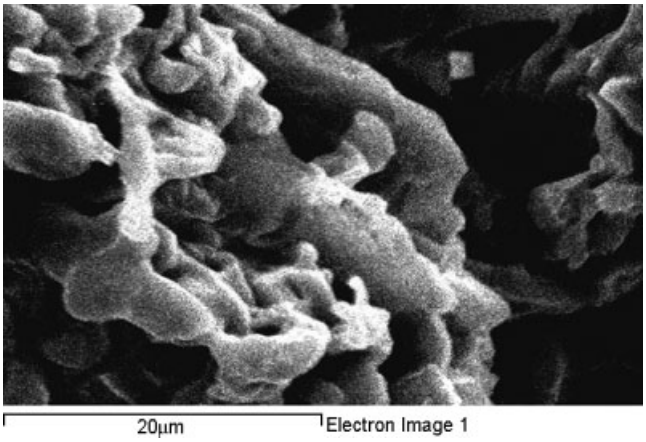

Figure 3. SEM image of the biodegradable scaffolds surface (with a pore size of 50–88 μm, ×3500).

The surface of the PLGA scaffold with respect to the osteoblast attachment was studied using a scanning electron microscope. As shown in Fig. 5, the PLGA scaffolds allowed for the adhesion and proliferation of the seeded osteoblasts through the pores during the 14-day *in vitro* culture period. Figure 6 also shows the attachment of the cells on the surfaces of the biodegradable scaffolds.

Alkaline phosphatase activity

The ALP activity of the osteoblasts cultured on the biodegradable scaffolds of 88–125 μm pore size increased during the culture period (14 days), as shown in Fig. 7. In contrast, the ALP activity of the osteoblasts grown on the 125–200 μm scaffolds decreased, while

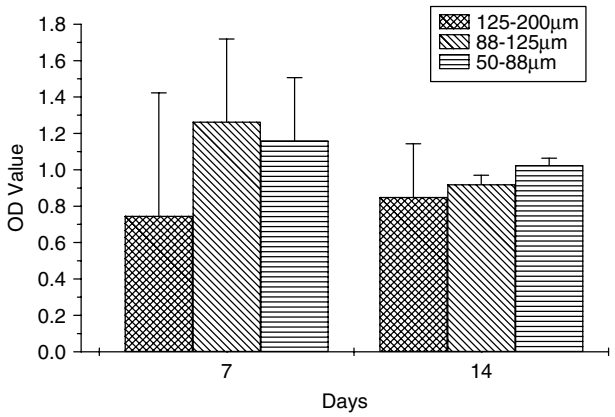

Figure 4. DNA assay result.

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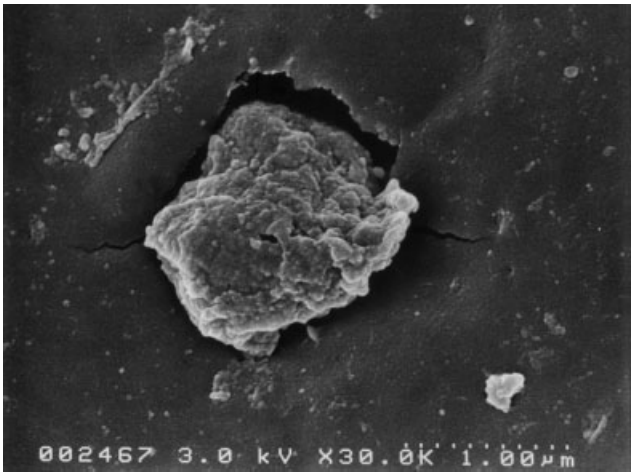

Figure 5. SEM photo of the cell/polymer constructs showing the growth of cells through the scaffold's pore (×30).

Figure 6. SEM photo of the cell/polymer constructs showing the attachment of the cells on the surfaces of the biodegradable scaffolds (×30).

the that of ones on 50–88 µm scaffolds did not show significant changes during the culture period. The osteoblasts on the 88–125 µm pore size scaffolds showed significantly higher levels of ALP activity compared to the osteoblasts on the 50–88 and 125–200 µm scaffolds during the period of culture.

Mineralization

The mineral deposition of cultured cells in the PLGA scaffolds was determined. The results in Fig. 8 showed that an increase of calcium levels, as well as abundant mineral deposition, was observed in the cultured MSCs. The histological observations of the alizarin red stain also showed the mineral deposition which was

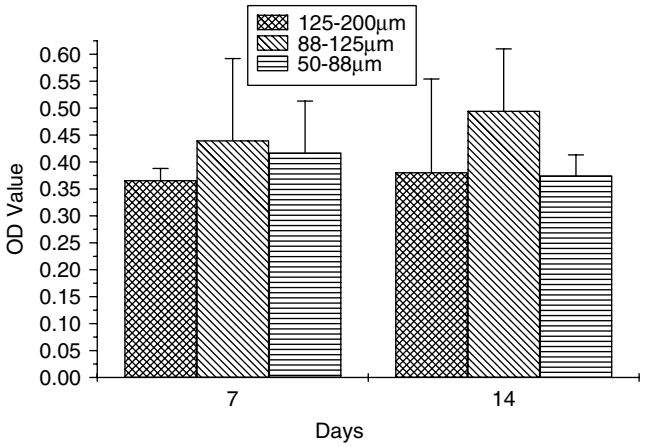

Figure 7. The alkaline phosphatase (ALP) activity of the osteoblasts cultured on PLGA scaffolds for 7 and 14 days.

indicated by red pigmentation in Fig. 9. In addition, the deposition on the PLGA scaffolds fabricated in this study gradually increased during the culture period for scaffolds of various pore sizes. The effect of pore size on the calcium deposition was found to be limited.

DISCUSSION

Tissue engineering of bone, like most tissue, requires three essential elements. First, cellular components must be present that are able to give rise to new structural tissue. Second, growth and differentiation factors must be available to guide the appropriate development of the cellular components. Third, a scaffolding matrix must be introduced to provide a substrate for cellular attachment, proliferation, and differentiation. This matrix may also serve to immobilize and orient the presentation of growth factors to the responding cells. In our current study, MSCs were used as the source of cellular component, and osteogenic medium was used as the growth

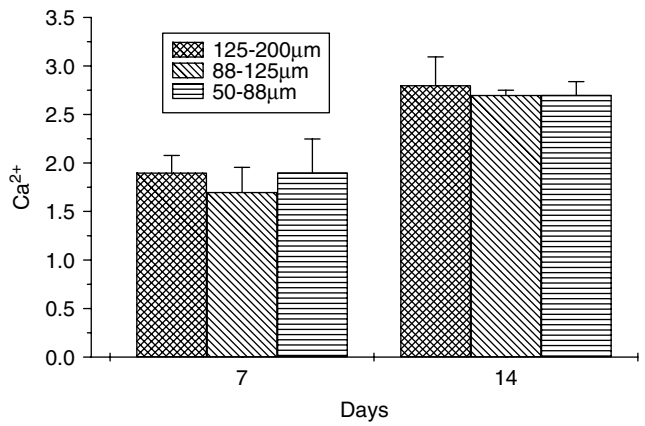

Figure 8. Calcium deposition in PLGA scaffolds.

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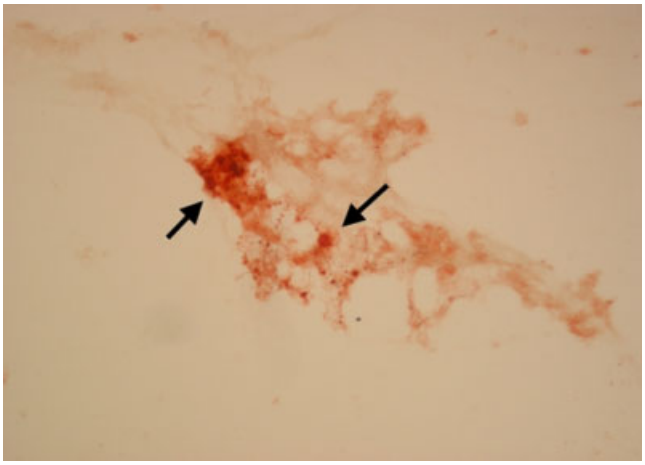

Figure 9. Histological evaluations of cell/polymer constructs 14 days after the cell cultures (↑: sites showing mineralization). This figure is available in colour online at www.apjChemEng.com.

and differentiation factor. A novel solvent-free method to fabricate biodegradable polymeric scaffolds was also developed to construct a carrier system for the tissue engineering of bone.

Various biomaterials have been used for tissue engineering. Biomaterials used for bone tissue engineering should maintain adequate mechanical strength, be osteoconductive, and degrade at a controlled rate to provide space for the formation of new bone. Polyesters of naturally occurring α-hydroxy acid, poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), and copolymers of poly(lactic-co-glycolic acid) (PLGA) have been extensively used to fabricate scaffolds for tissue engineering as they are nontoxic, elicit a minimal inflammatory response, and can be eventually absorbed without any accumulation in the vital organs.[10,11] Several techniques have been proposed in the literature to generate highly porous polymeric scaffolds. These methods include solvent casting/salt leaching,[17–20] fibrous fabric processing,[21–23] gas foaming,[15] emulsion freeze-drying,[14,24,25] three-dimensional printing,[26,27] and phase separation.[7,8,13,16,21,28–33] Most of these methods employ solvents for the manufacture of the scaffolds. However, residual solvents in the scaffolds may be harmful to transplanted cells or host tissues.[34]

In this study, solvent-free porous PLGA scaffolds were fabricated by the compression sintering and salt leaching method. As compared with other methods for fabricating biodegradable polymeric scaffolds, the compression sintering and salt leaching method has a number of advantages. First, the current process avoids the use of organic solvents. Residual organic solvents remaining in scaffolds may damage transplanted cells and surrounding tissues. Furthermore, exposure to organic solvents may inactivate biologically active factors.[34] The developed compression sintering/salt leaching process may minimize denaturation of the growth factors incorporated within the scaffolds. In addition, the residual solvent in the scaffolds may function as a plasticizer and make the polymer more ductile. Fabricating a scaffold without solvents will minimize the chance of degrading the mechanical properties of the biodegradable scaffolds.

During the fabrication (compression sintering) of polymer scaffolds, the formation of a homogeneous melt from powder particles involves two steps: First, the polymeric particles stick or fuse together at their points of contact around the NaCl particles. This fusion zone grows until the mass becomes a three-dimensional network, with relatively little density change. This is referred to as sintering.[35] Second, at some point in the fusion process, the network begins to collapse into the void spaces between the polymer and the salt particles. These spaces are filled with molten polymer that is drawn into the region by capillary forces. This is referred to as densification.[35] The NaCl is then encapsulated by the polymer to form a composite for the scaffolding. After sintering, the cylinder was submerged in distilled water for 48 h to leach out the NaCl. During the leaching process, the cylinders swelled and open channels were formed as a result of the swelling. The sodium chloride was thus released through a channel diffusion mechanism. Furthermore, the pore size of the fabricated scaffolds can be controlled by adjusting the size distribution of the NaCl used.

MSCs have the capacity for extensive replication with differentiation, and they possess a multilineage developmental potential allowing them to give rise to bone, cartilage, tendon, muscle, fat, and marrow stroma. Numerous investigators have described techniques for the isolation of human and animal MSCs from bone marrow and periosteum.[36–38] The isolation generally is based on density gradient centrifugation, and cell culturing techniques to separate the adherent MSCs from nonadherent cells. When MSCs are used for the treatment of bone defects, differentiation of the stem cells must occur after implantation of the construct. In an attempt to accelerate and enhance bone formation, several investigators have explored the use of predifferentiated osteoblasts in the tissue engineering of bone. Culturing MSCs with dexamethasone, ascorbic acid, and β-glycerophosphate directs the cells into the osteogenic lineage.[37,38] Theoretically, these predifferentiated osteoblasts can then be used to treat various bone defects.

In our current study, the osteogenic differentiation of the MSCs cultured on the solvent-free PLGA matrix has been well demonstrated by the expression of osteogenic genes, increasing ALP activity and calcium levels, and abundant mineral deposition after 14 days of growth. This finding suggests that the developed solvent-free biodegradable PLGA scaffold is a useful carrier system

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for the tissue engineering of bone, and may provide a potential method of treatment for bone defects.

CONCLUSIONS

This report has proposed a novel solvent-free method for the fabrication of three-dimensional scaffolds for tissue engineering. To manufacture a scaffold, polylactide-polyglycolide (PLGA) copolymers were premixed with sodium chloride particulates. The mixture was then compressed and sintered to form a cylinder. After sintering, the cylinder was submerged in water for 48 h to leach out the particulates. The scaffold with approximately 2×10^7 MSCs from the New Zealand rabbit was then cultured in an osteogenic culture medium for 14 days. The ALP activity, calcium level, and the mineral deposition of cultured cells in the PLGA scaffolds were determined. The results showed that an increase of ALP activity and calcium levels, as well as abundant mineral deposition, was observed in the cultured MSCs. In addition, scaffolds with pore sizes of 88–125 μm showed the most number of cells during the culture period. Developing a solvent-free biodegradable scaffold for bone cells may provide a potential method for the treatment of infected bone defects.

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