Poly(lactide-co-glycolide)/titania Composite Microsphere-Sintered Scaffolds for Bone Tissue Engineering Applications

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Abstract: The objective of this study was to synthesize and characterize novel three-dimensional porous scaffolds made of poly(lactic-co-glycolic acid) (PLGA)/nano-TiO_2-particle composite microspheres for potential bone repair applications. The introduction of TiO_2 component has been proven capable of largely enhancing mechanical properties of PLGA/TiO_2 microsphere-sintered scaffold ("PLGA/TiO_2-SMS"). In addition, composite nano-TiO_2 additives are capable of inducing an increased arrest of adhesive proteins from the environment, which benefits cell attachment onto the scaffolds. Osteoblast proliferation and maturation were evaluated by MTT assay, alkaline phosphatase (ALP) activity, and bony calcification assay. The results indicate that osteoblasts cultured on the composite scaffolds with different TiO_2 content (0, 0.1, and 0.3 g/1 g PLGA) display increased cell proliferation compared with pure PLGA scaffold. When cultured on composite scaffolds, osteoblasts also exhibit significantly enhanced ALP activity and higher calcium secretion, with respect to those on the pure PLGA scaffolds. Taken together, PLGA/TiO_2-SMSs deserve attention utilizing for potential bone-repairing therapeutics. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 93B: 84–92, 2010

Keywords: TiO_2 nanoparticles; PLGA; microspheres; scaffold; bone repair

INTRODUCTION

Tissue engineering, which provides an approach to aid in tissue regeneration, uses the basic principles of material technology and life science.[1–5] Scaffolds take a key role in tissue engineering strategy. Serving as a three-dimensional template for cell adhesion, proliferation, differentiation, and formation of a desired extracellular matrix (ECM), an ideal bone tissue engineering scaffold should comply with the following parameters: (1) osteoconductivity to guide bone around or inside the implant; (2) a biodegradability that matches the rates of ECM deposition and bone remodeling; (3) an adapted porosity that enables cell ingrowth; and (4) adequate mechanical properties.[6,7]

So far, various synthetic polymer materials such as poly(lactic acid), poly(glycolic acid), their copolymers [poly (lactic-co-glycolic acid) (PLGA)], poly-ε-caprolactone, and polyurethane have been used as scaffolds for tissue regeneration,[8–11] of which PLGA received great attention because of its good mechanical strength, biocompatibility, and tailored degradation rate.[12] However, PLGA chains lack functional groups, and each lactic acid residue contains a pendant methyl group, giving the surface a hydrophobic nature. One of the present trends in improving PLGA-based scaffold is to composite PLGA matrix with nanoparticles such as hydroxyapatite,[13] tricalcium phosphate (TCP),[14] and bioactive glass.[15]

Recently, TiO_2 particles have drawn more attention in the biomedical field. TiO_2 powders are effective in apatite formation on PLGA/TiO_2 composite surface in simulated body flu-

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ids (SBF), which is believed to be a prerequisite for bioactivity.[16] Liu et al.[17] found that TiO_2 nanoparticles effectively enhanced cell attachment and proliferation. Goto et al.[18] also found that TiO_2-containing bone cement could not only allow regulation of the setting time and the handling of bone cement but also improve the osteoconductivity *in vitro* and *in vivo*. Nanosized titania can also promote protein absorption and osteoblasts adhesion.[17] Therefore, nano-TiO_2 could be a potential material for bone repair applications.

The ideal drug loading efficiency and the drug dosage optimization make PLGA microsphere as an excellent controlled release carrier. Kang et al.[19] developed an injectable PLGA microsphere scaffold for cartilage tissue repair by means of minimally invasive surgical procedures. In addition, porous PLGA microcarriers have also been used for injectable cell therapy.[20] Borden et al.[12] developed a microsphere-sintered scaffold in which polymers such as PLGA were fabricated into microspheres by means of single emulsion techniques. By the action of heating and solvent, PLGA microspheres formed a scaffold with good mechanical properties. Compared with traditional methods such as solvent casting/particulate leaching and thermally induced phase separation, the mechanical properties of PLGA-sintered microsphere scaffold were remarkably improved.[12] Moreover, combining with the advantage of PLGA microsphere, PLGA-sintered microsphere scaffolds are also promising multifunctional vehicles for drug/protein delivery and tissue engineering.[21]

In this study, PLGA/TiO_2-sintered microsphere scaffolds were produced. The absorption of an important multiadhesive matrix protein—fibronectin (FN), and the proliferation and maturation of osteoblasts on these composite scaffolds were evaluated. The ultimate objective of this work is to develop a potential bone repair scaffold with good mechanical properties and beneficial cytocompatibility. This new PLGA/TiO_2 composite-sintered microsphere scaffold is abbreviated as "PLGA/TiO_2-SMS" system.

MATERIALS AND METHODS

Materials

PLGA (lactic/glycolic 1:1; M_w 31,000 Da; inherent viscosity 0.30 dL/g in chloroform at 30°C) was purchased from Daigang Biomaterials (Jinan, China). Poly(vinyl alcohol) (PVA) and human FN were obtained from Sigma (Singapore). TiO_2 nanoparticles (Aeroxide-P25) were purchased from Degussa (Shanghai, China) with a mean primary size of 21 nm, specific surface area of 50 m^2/g, and density of 3.966 g/cm^3. The crystalline structure of TiO_2 nanoparticles consist of approximately 70% anatase and 30% rutile.

Preparation of PLGA/TiO_2 Composite Microspheres and Scaffolds

TiO_2 (0, 0.1, and 0.3 g) was mixed with PLGA (1 g) solution in methylene chloride (5 mL) by stirring until uniform suspension formed, and then, the mixture was added dropwise to a stirred 1% PVA solution. The resultant emulsion was kept at 37°C under stirring at a speed of 200 rpm for 12 h to allow solvent to evaporate completely. PLGA/TiO_2 microspheres were finally washed with deionized water and then dried. The round microspheres were collected by using sieves with the sizes of 335 and 154 μm and stored in a dessicator for future use.

PLGA/TiO_2-SMS with 0, 0.1, and 0.3 g nano-TiO_2 [abbreviated as PLGA-SMS, PLGA/TiO_2(10)-SMS, and PLGA/TiO_2(30)-SMS, respectively] were fabricated by pouring microspheres into a cylindrical silica gel mold (diameter = 10 mm, height = 20 mm) and heated at 90°C for 2 h. After the molds cooled down to the room temperature, the samples were removed from the molds.

Static Contact Angles and Surface Roughness

Nano-TiO_2 particles were mixed with a solution containing 2 g PLGA in 10 mL methylene chloride according to a 1:10 or 3:10 TiO_2 to PLGA weight ratio. The mixture was vortexed until uniform suspension formed and was poured into a glass dish. Subsequently, PLGA/TiO_2 composite film was removed when the solvent evaporated completely.

The static contact angles of PLGA and PLGA/TiO_2 films (1 × 1 cm) were measured with a contact angle analyzer (First Ten Ångstroms, Virginia, USA) using the sessile drop technique. The measurements were performed at room temperature with deionized water as the probe liquid. Twenty-five-microliter liquid droplets were deposited onto the sample surface through a gauge dispensing needle at a rate of 5 μL/s. Each contact angle reported here is an average of at least five measurements, and the contact angles were determined with direct optical images by a camera. Surface roughness of PLGA and PLGA/TiO_2 films were determined by Optical profiler (Wyko NT9000, USA).

Density and Porosity Determination of Scaffolds

Density and porosity of PLGA-SMS and PLGA/TiO_2-SMS were determined following the method described in reference.[22] In brief, ethanol was used as the liquid phase and kept at 25°C. A bottle filled with ethanol was weighed (W1). Then, a scaffold sample weighing W_S was immersed into the bottle and weighed (W2). ρ is the density of ethanol at 25°C The size of the cylindrical scaffold, including radius (R) and height (H), was measured. The porosity (P) and density (D) were calculated using the equations as follows:

$$P = 1 - ((W1 - W2 + Ws)/\rho)/((\pi \times R^2) \times H) \quad (1)$$

$$D = Ws/((\pi \times R^2) \times H) \quad (2)$$

Mechanical Testing

The compressive strength and compressive modulus of the cylindrical scaffolds (diameter = 10 mm, height = 20 mm; n = 6) were measured using a universal material testing

machine (Instron 5567, Instron, USA) at a crosshead speed of 5 mm/min for compressive strength tests.

Protein Adsorption

The PLGA/TiO_2 and PLGA microspheres (50 mg) were first incubated in 50 μg/mL human fibronectin [FN/phosphate-buffered saline (PBS)] solution (500 μL) or 150 μg/mL bovine serum albumin (BSA/PBS) solution (500 μL) for 6 h on shaker (25 rpm), respectively.[12] After thorough rinsing with PBS, the microspheres were homogenized in 1% sodium dodecyl sulfate solution and then subjected to centrifuge at 4°C for 15 min. The total protein in the supernatant was quantified using Bradford Reagent assay (Sigma–Aldrich, USA) and MicroBCA™ protein assay (Pierce, Rockford, IL).

Cell Culture and Seeding

Human fetal osteoblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified eagle's medium (DMEM)/Ham's F12 (1:1) cultural medium supplemented with 2.5 m*M* L-glutamine, 0.3 mg/mL G418, and 10% (v/v) fetal bovine serum (FBS). All these cell culture-related reagents were purchased from Gibco (Invitrogen, Singapore). The fabricated cylindrical scaffolds (diameter = 5 mm and height = 3 mm) were sterilized by 70% ethanol for 2 h followed by PBS wash. All the scaffolds were prewetted in the culture medium for 24 h. Fifty microliters of cell suspension (2×10^8 cells/mL) were seeded on every scaffold. The cells were allowed to adhere to the scaffolds for 3 h, and then, 750 μL of culture medium was added to each scaffold. The cell–scaffold complexes were cultured at 39°C in a humidified incubator of 5% CO_2 following the manufacturer's instruction.

Cell Viability

Cell viability was evaluated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Roche Diagnostics, Germany) assay. In brief, cylindrical PLGA-SMSs and PLGA/TiO_2-SMSs (diameter = 5 mm and height = 3 mm) were placed onto each well (24-well tissue culture polystyrene plates). After treating the cells with scaffolds for 3, 7, and 14 days, the supernatant medium was replaced by MTT diluted 1:20 (v/v) with DMEM and incubated for 3 h. The level of dye formed was then measured using Elisa reader at days 3, 7, and 14. The cell viability on the scaffolds was detected at day 14 using a "Live/Dead" assay (calcein/ethidium bromide; Molecular probes, Invitrogen, Singapore), following the manufacturer's instruction. Cell attachment was determined using Hoechst assay (Hoechst 33258, Molecular Probes, Invitrogen), following the manufacturers' instructions. Through the measured DNA quantities, the exact numbers of the committed cells could be counted using a conversion rate of 6.6 pg DNA per cell. Cell attachment efficiency was calculated using the equations as follows:

$$\mathrm{CAE}\ (\%) = (N_6/N_0) \times 100$$

where CAE, N_0, and N_6 denote cell attachment efficiency, the number of cells seeded on the scaffolds, and the number of cells after 6 h of culture.

Osteogenic Analysis

Osteoblasts that had been prewashed with PBS were lysed in 0.5 mL PBS containing 0.1*M* glycine, 1 m*M* $MgCl_2$, and 0.05% Triton X-100. The lysate solution was incubated with *p*-nitrophenyl phosphate solution at 37°C for 30 min and subjected to a spectrophotometer on which the absorbance at 405 nm was measured and recorded to indicate alkaline phosphatase (ALP) concentration at days 3, 7, and 14.[23] A semiquantitative Alizarin red-based assay of mineralization by osteoblasts was performed as described previously.[24]

Statistical Analysis

Experiments were repeated three times, and results were expressed as means ± standard deviations. Statistical significance was calculated using one-way analysis of variance. Comparison between each two means was determined using the Tukey test, and statistical significance was defined as $p < 0.05$.

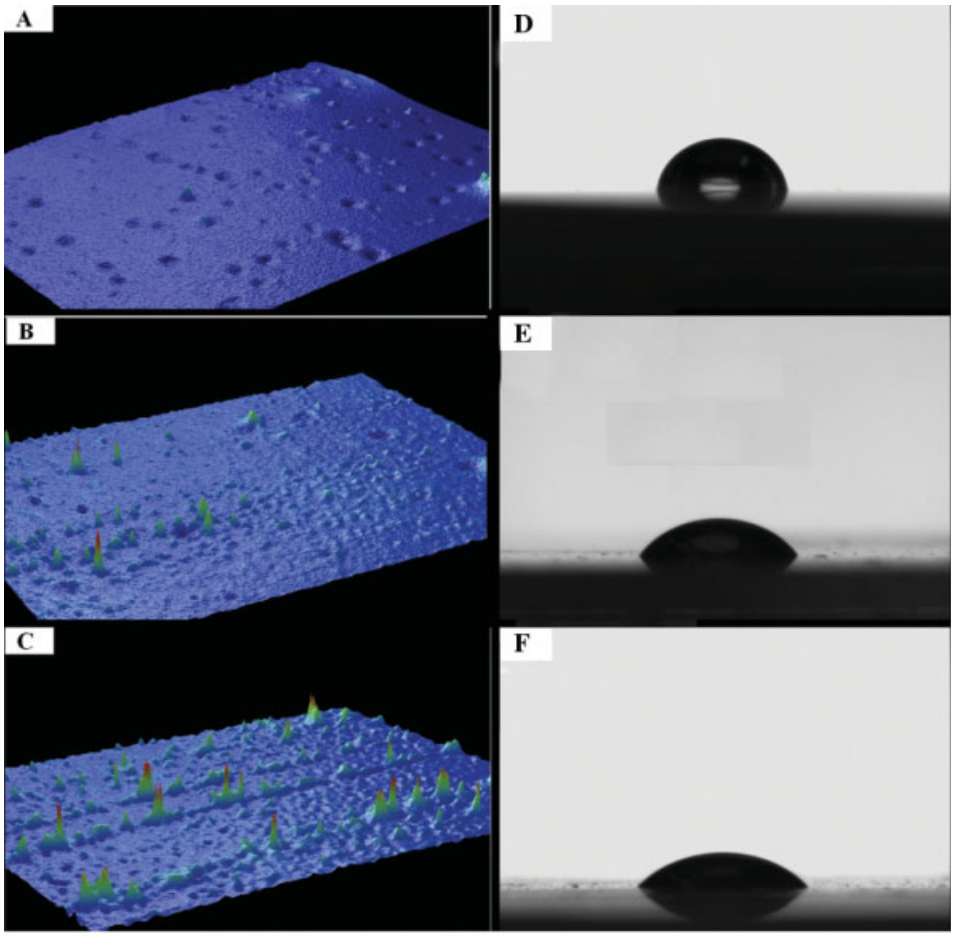
RESULTS

Surface Roughness and Wettability

As shown in Figure 1, all the contact angles of different PLGA/TiO_2 composite surfaces were significantly declined, which indicated that the hydrophilicity of material surface increased by addition of TiO_2. When the TiO_2 content is 0.3 g/g PLGA, the contact angle was approximately 53.9°. The surface roughness of PLGA and PLGA/TiO_2 composite films is also displayed in Figure 1. Lots of concave pores were present on the surface of PLGA film, which may be due to evaporation of methylene chloride. In contrast, it is observed that a number of "humps" as a result of TiO_2 particle enrichment were exhibited on the surface of PLGA/TiO_2 films, which indicated that addition of TiO_2 particles into PLGA bulks increased the surface roughness to a large extent.

Scaffolds Morphology

Figure 2 shows the morphology of PLGA-SMS and PLGA/TiO_2 (30)-SMS. The scaffolds were built by microspheres, and all types of PLGA-based microspheres maintained in spherical shape, among which the TiO_2-containing ones displayed visible rough surfaces. These morphologies were



	Surfece Roughness (Ra, nm)	Contact Angle (°)
PLGA	12.2±2.9	73.6±2.6
PLGA/TiO_2(10)	28.0±5.2	60.2±3.0
PLGA/TiO_2(30)	47.9±7.0	53.9±1.8

Ra: Roughness average

Figure 1. Surface morphologies and contact angles of PLGA (A and D), PLGA/TiO_2(10) (B and E), and PLGA/TiO_2(30) (C and F). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

due to great nano-TiO_2 conglomeration enrichment on their surfaces.

Porosity and Density

As shown in Figure 3, all groups of scaffolds have the similar porosity (between 30 and 40%). A TiO_2-dependent increase in the density of scaffolds was observed, and the density of PLGA/TiO_2-SMS with TiO_2 particles of 0.1 g/g PLGA and 0.3 g/g PLGA was (0.83 ± 0.05 g/cm^3) and (1.13 ± 0.17 g/cm^3), which was significantly higher than that of PLGA-SMS.

Mechanical Properties

Figure 4 shows compressive strength and compressive modulus of the PLGA/TiO_2 and PLGA scaffolds. PLGA and PLGA/TiO_2 microspheres were produced by means of a single emulsion solvent evaporation technique. Compared with PLGA-SMS, PLGA/TiO_2-SMS showed significantly greater compressive strength and compressive modulus. The compressive strength and compressive modulus of PLGA/TiO_2(10)-SMS and PLGA/TiO_2(30)-SMS were 4.76 ± 0.52 and 147.25 ± 11.27 MPa and 6.70 ± 0.36 and 222.33 ± 20.16 MPa, respectively. On the contrary, the compressive strength and compressive modulus of PLGA scaffolds were 4.15 ± 0.27 and 111.18 ± 19.89 MPa, respectively.

Protein Adhesion and Cell Attachment

DMEM with 10% FBS, 1% BSA, and FN were used to evaluate protein adsorption onto scaffolds (Figure 5). Quantitative measurements indicated that the amounts of all proteins in PLGA/TiO_2-SMS were greater than those in

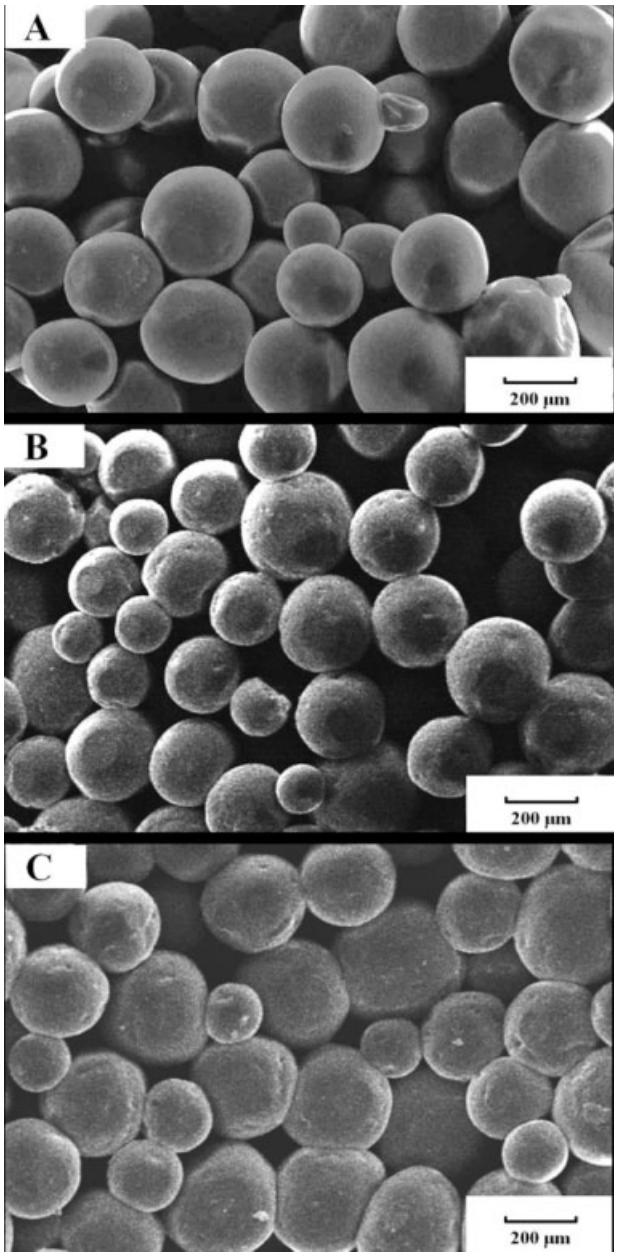


Figure 2. SEM images of PLGA-SMS (A), PLGA/TiO_2(10)-SMS (B), and PLGA/TiO_2(30)-SMS (C).

PLGA-SMS. Specifically, compared with PLGA-SMS, FN arrest was twice greater on PLGA/TiO_2(30)-SMS. Compared with PLGA-SMS, osteoblast adhesion was significantly greater on PLGA/TiO_2-SMS surface after 6 h. Actually, osteoblast adhesion increased >60% on PLGA/TiO_2(30)-SMS compared with PLGA-SMS.

Osteoblastic Proliferation and Maturation

The fluorescence microscope images (Figure 6) showed visualized cell viability on the scaffolds. All types of scaffolds with cells were stained by "Live/Dead" assay after 14 days of culture *in vitro*, and cell number was also detected by DNA quantification assay. A significantly

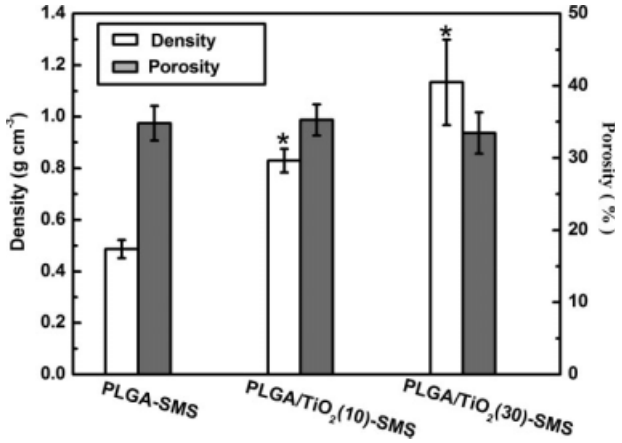


Figure 3. Porosity and density of PLGA-SMS and PLGA/TiO_2-SMS. (*) indicates statistical significance when compared with PLGA-SMS.

greater number of cells was observed when cells were cultured on the PLGA/TiO_2(30) scaffold [$(2.71 \pm 0.32) \times 10^7$] compared with cells on the PLGA/TiO_2(10)-SMS [$(2.12 \pm 0.19) \times 10^7$] and PLGA-SMS [$(1.66 \pm 0.25) \times 10^7$]. On all groups of scaffolds, cells grew well and proliferated on microspherical surface on day 14. Osteoblasts were seeded on PLGA-SMS and PLGA/TiO_2-SMS over 14-day culture period *in vitro*. Cell proliferation in the scaffolds was analyzed using MTT assay after 3, 7, and 14 days of culture (Figure 7). On the third day, cell proliferation within the PLGA/TiO_2-SMS was significantly higher than that of PLGA-SMS. Up to day 7, PLGA/TiO_2(30)-SMS exhibited remarkable cell proliferation, which was nearly 20 and 30% higher than that of PLGA/TiO_2(10)-SMS and PLGA-SMS, respectively.

ALP content (Figure 8) was analyzed after 3, 7, and 14 days of cell culture in the scaffolds. The ALP activity of the scaffolds increased continuously during the culture period for all groups. After 14 days of culture, the osteoblasts on the PLGA/TiO_2(30)-SMS showed significantly higher levels of

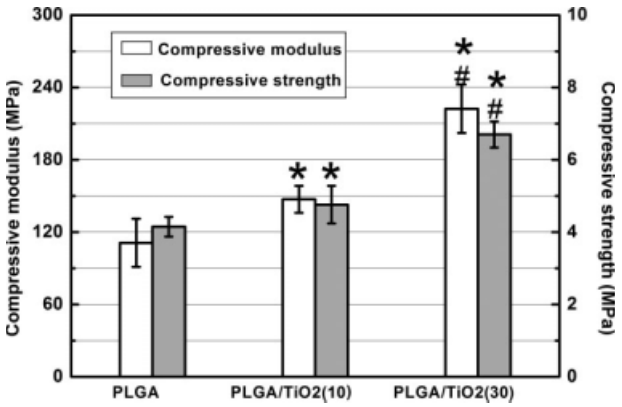


Figure 4. Mechanical properties evaluation in compression for PLGA-SMS (A), PLGA/TiO_2(10)-SMS, and PLGA/TiO_2(30)-SMS. (*) and (#) indicate statistical significance when compared with PLGA-SMS and PLGA/TiO_2(10)-SMS.

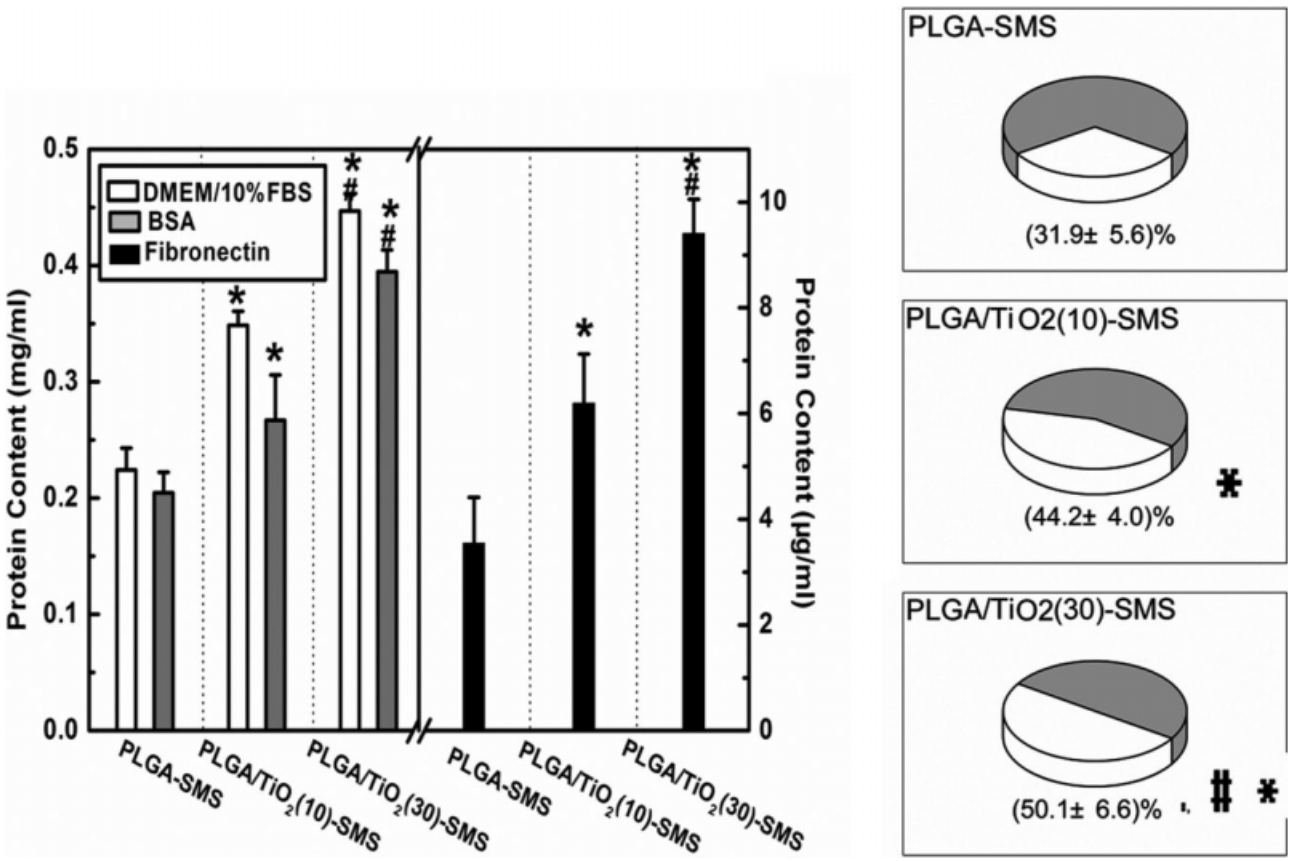


Figure 5. Adsorbed human fibronectin (FN) and bovine serum albumin (BSA) in PLGA/TiO_2-SMS and PLGA/TiO_2-SMS, and cell attachment on PLGA/TiO_2-SMS and PLGA/TiO_2-SMS. (*) and (#) indicate statistical significance when compared with PLGA-SMS and PLGA/TiO_2(10)-SMS.

ALP activity compared with other groups. A critically important function of osteoblasts is participating in biological mineralization. The calcium deposition by osteoblasts was accessed by a semiquantitative Alizarin red-based assay (Figure 9). The results showed that the calcium content of cell secretion on the scaffolds increased continuously during the culture period. Af-

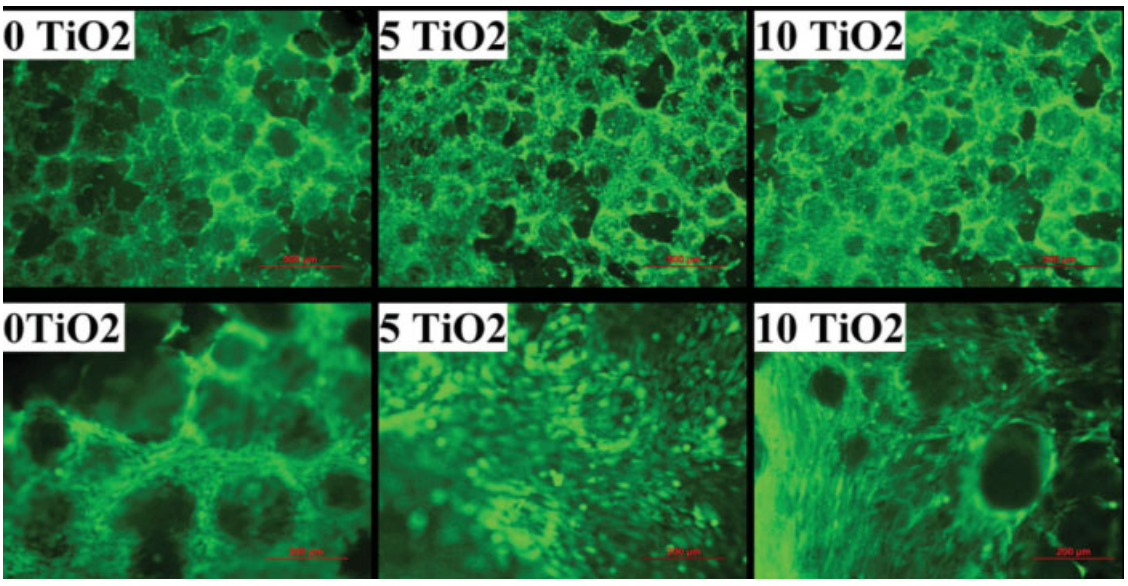


Figure 6. Live/Dead assay of cell growth on the PLGA-SMS PLGA/TiO_2(10)-SMS and PLGA/TiO_2(30)-SMS for 14 days. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

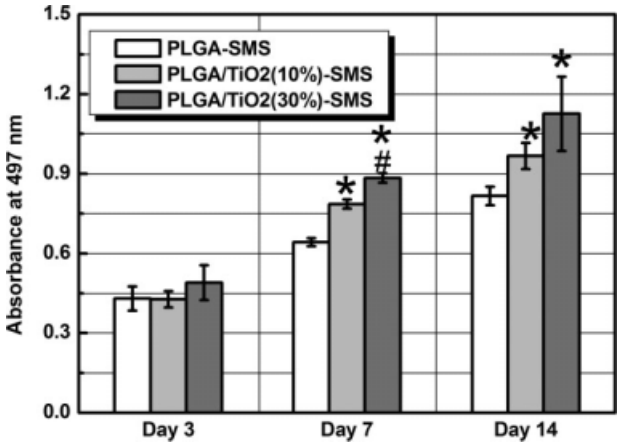


Figure 7. Cell proliferation evaluation by MTT on the PLGA scaffold, PLGA/TiO_2(10), and PLGA/TiO_2(30). (*) and (#) indicate statistical significance when compared with PLGA-SMS and PLGA/TiO_2(10) scaffolds.

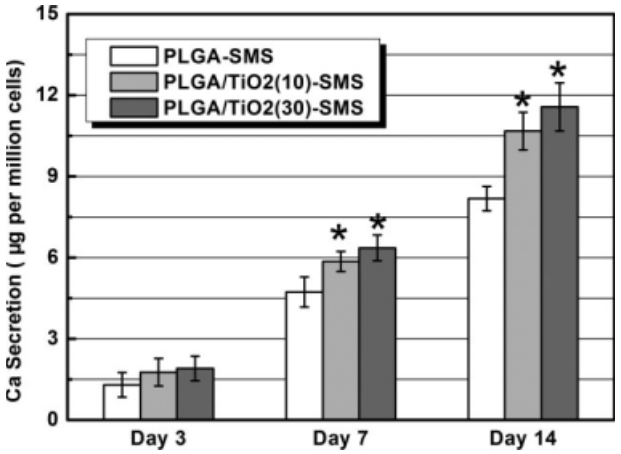


Figure 9. Calcium deposition of osteoblasts on PLGA-SMS and PLGA/TiO_2-SMS for 14 days. (*) indicates statistical significance when compared with PLGA-SMS.

ter 7 and 14 days of culture, the calcium deposition on PLGA/TiO_2(30)-SMS scaffold was at higher levels than that on PLGA and PLGA/TiO_2(10)-SMS scaffolds.

DISCUSSION

PLGA-based biodegradable polymeric materials have been extensively used in tissue engineering and controlled release fields because of their excellent processability, mechanical properties, and controlled degradation.[25,26] As a synthetic polymer, PLGA lacks functional groups, thereby the improvement of its biocompatibility is demanded. Many approaches have been carried out to enhance the biofunctionality of PLGA.[27,28] In this study, nano-TiO_2 particles were incorporated into PLGA bulk to improve its mechanical behavior and biocompatibility.

Microsphere-sintered technique was used to manufacture PLGA/TiO_2 composite scaffolds. The microspheres with 0.1 g TiO_2/g PLGA and 0.3 g TiO_2/g PLGA were evaluated. It has been reported by Torres et al.[16] that the more titania particles the sample contained, the better was its apatite-forming ability in SBF. The content of TiO_2 was controlled below 0.4 g/g PLGA because the composite microspheres were deformed during emulsion process when the TiO_2 content was increased to 0.4 g/g PLGA. The deformed microspheres could influence properties and structure of the composite scaffold.

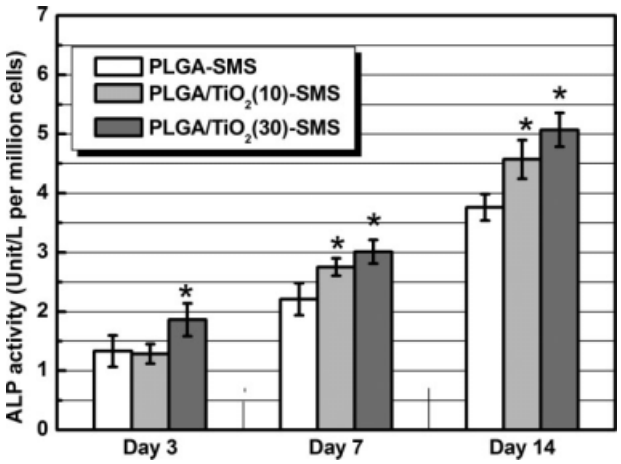


Figure 8. Alkaline phosphatase activity of osteoblasts cultured on PLGA-SMS and PLGA/TiO_2-SMS for 14 days. (*) indicates statistical significance when compared with PLGA-SMS.

The clinical fates of implants, substitute materials, and scaffolds used in bone tissue engineering strategies critically depend on their mechanical and biological properties. By incorporating TiO_2 nanoparticles into PLGA bulk, PLGA/TiO_2-SMSs exhibit similar mechanical properties as cancellous bone (compressive modulus: 50–500 MPa, compressive strength: 2–12 MPa), which caters for the need on mechanical behavior for cancellous bone repair.[29]

In addition to enhancing mechanical properties of PLGA scaffolds, TiO_2 particles also assume active role in improving biocompatibility of the scaffolds. As the results of MTT assay (Figure 7) showed, osteoblast proliferation on TiO_2-containing scaffolds was significantly higher compared with TiO_2-free scaffolds. TiO_2 nanoparticles can increase protein absorption and subsequent osteoblast adhesion. This is in accordance with the report that TiO_2-coated PLGA film promoted the attachment and the proliferation of human dermal fibroblasts and rat cortical neural cells.[17,18]

Further investigation was conducted to analyze the protein adhesion on the scaffolds. As shown in Figure 6, Ti-containing PLGA scaffold absorbed more environmental proteins such as FN and BSA. As well known, FBS contains many ECM proteins such as FN. FN is an abundant multiadhesive matrix protein, which is crucial in cell adhesion through Arg-Gly-Asp (RGD) sequence.[30,31] As shown in Figure 5, there is significant statistical difference between cell adhesion to pure PLGA-SMS and PLGA/TiO_2-SMS, suggesting that PLGA and nano-TiO_2 particles

composite scaffolds promoted osteoblast adhesion. In addition, the maximum adhesion of osteoblasts occurred on PLGA/TiO_2(30)-SMS. From the results of protein absorption and cell adhesion assays, we can conclude that the increased cell adhesion on TiO_2-containing scaffolds could be mainly attributed to more functional proteins such as FN arrested by the scaffolds from the environmental medium before cell contact.

As an early osteogenic maker, ALP activity was remarkably higher for the osteoblasts on PLGA/TiO_2-SMS (Figure 8). ALP is expressed mainly on cell surfaces or in matrix vesicles. It specifically degrades the organic phosphoesters in bone and cartilage, which inhibits cartilage mineralization and promotes the calcium deposition in bone.[32] The measurement of calcium deposition is important for osteogenesis because calcium is the main component of extracellular bone matrix.[33,34] In this study, the osteoblasts on PLGA/TiO2-SMS also showed significantly higher calcium deposition (Figure 9).

Taken together, in this study, PLGA/TiO_2-SMSs were fabricated by single emulsion and microsphere-sintered techniques. PLGA/TiO_2-SMS exhibited adapted surface properties for FN arrest and cell attachment and showed mechanical properties similar to those of cancellous bone. In addition, PLGA and TiO_2 composite scaffolds could effectively enhance osteoblast proliferation and maturation. Therefore, PLGA/ TiO_2-SMS is a promising scaffold for bone repair.

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