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In Vitro Mineralization by Preosteoblasts in Poly(D, L-lactide-co-glycolide) Inverse Opal Scaffolds Reinforced with Hydroxyapatite Nanoparticles

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Abstract

Inverse opal scaffolds made of poly(D, L-lactide-co-glycolide) (PLGA) and hydroxyapatite (HAp) were fabricated using cubic-closed packed (ccp) lattices of uniform gelatin microspheres as templates and evaluated for bone tissue engineering. The scaffolds exhibited a uniform pore size ($213 \pm 4.4 \mu\text{m}$), a porosity of $\sim 75\%$, and an excellent connectivity in three dimensions. Three different formulations were examined: pure PLGA, HAp-impregnated PLGA (PLGA/HAp), and apatite (Ap)-coated PLGA/HAp. After seeding with preosteoblasts (MC3T3-E1), the samples were cultured for different periods of time and then characterized by X-ray microcomputed tomography (micro-CT) and scanning electron microscopy to evaluate osteoinductivity in terms of the amount and spatial distribution of mineral secreted from the differentiated preosteoblasts. Our results indicate that preosteoblasts cultured in the Ap-coated PLGA/HAp scaffolds secreted the largest amount of mineral, which was also homogeneously distributed throughout the scaffolds. In contrast, the cells in the pure PLGA scaffolds secreted very little mineral, which was mainly deposited around the perimeter of the scaffolds. These results suggest that the uniform pore structure and favorable surface properties could facilitate the uniform secretion of extracellular matrix from cells throughout the scaffold. The Ap-coated PLGA/HAp scaffold with uniform pore structure could be a promising material for bone tissue engineering.

Keywords

hydroxyapatite; preosteoblasts; osteoinductivity; X-ray microcomputed tomography (micro-CT)

Introduction

Scaffold design is important in tissue engineering due to its role in supporting cell attachment and growth, assisting exchanges of nutrients/oxygen and metabolite wastes, and eventually facilitating the formation of a functional tissue/organ. Many parameters need to be considered for scaffold design: pore size, porosity, interconnectivity, surface properties, and mechanical properties, among others.

For bone tissue engineering, hydroxyapatite (HAp), a major inorganic component in natural bone, has been often used due to its osteoinductivity, high mechanical strength, and biocompatibility.¹ Therefore, much attention has been paid to the impregnation of a scaffold

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matrix with HAp nanoparticles and the decoration of a scaffold surface with apatite (Ap) using a simulated body fluid (SBF).² Many research groups have demonstrated that incorporation of HAp nanoparticles and Ap coating could enhance preosteoblast differentiation and mineral secretion.³ However, most of these studies were limited to the evaluation of gross properties (e.g., overall mineral content). Few studies have discussed the microscopic distribution of mineral throughout the scaffolds. The uniform distribution of cells and extracellular matrix (ECM) in the scaffold with suitable mechanical properties is critical for successful bone tissue engineering because a region devoid of cells and/or ECM might become a defect after the formation of bone.⁴ Therefore, the scaffold should have a uniform pore structure and induce homogeneous production of the appropriate organic (e.g., collagen) and inorganic (e.g., hydroxyapatite mineral) ECM throughout the scaffold. From this point of view, inverse opal structure is excellent scaffold platform for bone tissue engineering.

We recently demonstrated the fabrication of a chitosan scaffold with an inverse opal structure (i.e., inverse opal scaffold) using a cubic-close packed (ccp) lattice of uniform polymer microspheres as the template.⁵ However, the chitosan inverse opal scaffold is not appropriate for bone tissue engineering because of its low mechanical strength. Therefore, we further developed three new kinds of inverse opal scaffolds composed of poly(D, L-lactide-co-glycolide) (PLGA) and HAp: *i*) PLGA alone, *ii*) HAp-impregnated PLGA (PLGA/HAp), and *iii*) Ap-coated PLGA/HAp scaffolds. We hypothesized that the HAp-impregnated inverse opal scaffold could provide a more favorable environment for bone tissue engineering due to its uniformity in porosity and surface/mechanical properties, eventually leading to uniform secretion of organic and inorganic matrix throughout the scaffold. In this work, preosteoblasts (MC3T3-E1), the most commonly used cell line for the study of bone tissue engineering, were seeded into the three types of inverse opal scaffolds and induced to differentiate and secrete mineral. The production of mineral (inorganic ECM) serves as an indicator of osteoblastic differentiation of the preosteoblast.⁶ To quantitatively evaluate the spatial distribution and amount of secreted mineral, X-ray microcomputed tomography (micro-CT) was used to examine the three-dimensional (3D) density and architecture of mineral in a non-invasive manner.⁷ To our knowledge, this is the first microscopic evaluation of the spatial distribution of mineral secreted from differentiated preosteoblasts onto inverse opal structures using micro-CT.

Experimental Section

Materials

Gelatin (Type A, from porcine skin, Sigma), sorbitan monooleate (Span[®] 80, Sigma), and toluene (99.8 %, Sigma-Aldrich) were employed to produce the uniform microspheres using a simple fluidic device. Poly(D, L-lactide-co-glycolide) (PLGA, lactide 75: glycolide 25, $M_w \approx 66,000$ -107,000, Sigma) and hydroxyapatite (HAp) nanopowder (<200 nm in size, Aldrich) were used as the materials for the scaffolds. All of the chemicals for the preparation of 10 times concentrated simulated body fluid (10×SBF) were obtained from Sigma-Aldrich. Ethanol (99.5%), methanol (99.0%), and 1,4-dioxane (99.8%) were also purchased from Sigma-Aldrich. The water used in all reactions was obtained by filtering through a set of Millipore cartridges (Epure, Dubuque, IA).

Preparation of inverse opal scaffolds

Uniform gelatin microspheres and PLGA inverse opal scaffolds were fabricated by modifying our previous method.⁵ A 50-mL centrifuge tube with a methanol dispersion of gelatin microspheres (~1.5 wt%) of $225.8 \pm 2.4 \mu\text{m}$ in size was sonicated for 10 s while the cap was closed, and placed on an orbital shaker set to 80 rpm for 1 h. The tube was gently

tapped with a finger for about 1 min to form a ccp lattice. The resultant ccp lattice was carefully placed in an oven heated at 65 °C for 1 h to make connections between the gelatin microspheres. After cooling to room temperature for 30 min, the ccp lattice pellet was harvested with a spatula, placed on a piece of filter paper to remove the methanol via evaporation, and then infiltrated with the PLGA solution in 1,4-dioxane (20 wt%). After removing the excess PLGA solution with filter paper, the ccp lattice pellet with PLGA solution was frozen (-20 °C) for 5 h and then lyophilized in a freeze-dryer (Labconco Co., USA) overnight. The ccp lattice pellet with freeze-dried PLGA was placed in ethanol for 5 min under vacuum and subsequently put in 900 mL of water heated at 45 °C for 3 h with stirring to remove the gelatin lattice. To fabricate PLGA/HAp scaffolds, the PLGA solution with HAp nanopowder (20 wt%) was sonicated for 30 min, vortexed for 1 min, and then infiltrated into the voids of the gelatin ccp lattice. To fabricate Ap-coated PLGA/HAp scaffolds, 10×SBF was used to deposit Ap on the PLGA/HAp scaffolds. Specifically, ten scaffolds were soaked in 100 mL of the SBF for 2 h under gentle stirring on an orbital shaker and then rinsed with water.⁸ All of the inverse opal scaffolds were sterilized by immersing in 70% ethanol for 2 h prior to cell seeding.

To determine the mechanical properties of the scaffolds, cylindrical specimens ($n = 3$ per group) were prepared using a poly(dimethyl siloxane) mold. Initial thickness (L_0) and cross-sectional area of the scaffolds were as follows: 1.66 ± 0.01 mm and 38.0 ± 1.45 mm² for PLGA alone, 1.80 ± 0.12 mm and 37.48 ± 0.26 mm² for PLGA/HAp, and 1.97 ± 0.18 mm and 39.90 ± 0.24 mm² for Ap-coated PLGA/HAp scaffolds. The scaffolds were sandwiched between two glass slides with unconfined compression using a custom-built testing frame. Compression was applied at a crosshead speed of 0.01 mm/s (~0.6% per second) at ambient temperature and humidity. Load and displacement data were collected using a computerized data acquisition system (Labview 12.0, National Instruments, USA). Engineering stress was calculated as the load divided by the sample cross-sectional area. Strain was calculated as change in displacement divided by the original thickness of the sample. Modulus was calculated by linear regression using data from the linear portion of the stress-strain plot.

Cell culture with the inverse opal scaffolds

Mouse calvaria-derived, preosteoblastic cells (MC3T3-E1; ATCC CRL-2593) were used for cell-based experiments. The cells were maintained in alpha minimum essential medium (α -MEM, without L-ascorbic acid, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% antibiotics (containing penicillin and streptomycin, Invitrogen). Prior to cell seeding, the inverse opal scaffolds were wetted and sterilized by immersion in 70% ethanol for 2 h, washed with PBS (Invitrogen) three times and stored in culture medium. Approximately 1.5×10^5 cells were used for seeding onto each scaffold using a spinner flask (125 mL capacity, Proculture™, Corning) at 65 rpm for 2 h at 37 °C and 5% CO₂. The cell-seeded scaffolds were then transferred to 24-well plates and cultured in osteogenic differentiation medium supplemented with 300 μ M L-ascorbic acid and 10 mM β -glycerol phosphate (Sigma-Aldrich). The cultures were maintained in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂ and the medium was changed every other day.

Cell proliferation and metabolization were measured at 0, 5, 14 and 28 days post cell seeding using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, with $n = 3$ per group per time point. MTT is a tetrazole that can be metabolized and reduced to purple formazan in live cells. Assays were carried out in 12-well plates: 40 μ L of MTT solution in PBS (5 mg/mL) was added to each well and incubated at 37 °C for 6 h and culture medium was withdrawn. 1 mL of isopropanol was then added to each well to completely dissolve formazan crystals throughout the scaffold. Optical density was

measured at 560 nm using a spectrophotometer (Infinite 200, TECAN). All final data were normalized to the dry weight of the scaffold.

SEM and micro-CT analysis

SEM (Nova NanoSEM 2300, FEI) was used to characterize the scaffolds, as well as the morphologies and behaviors of the cells grown in the scaffolds ($n = 3$ per group per time point). Prior to imaging, the cells were fixed and samples were dehydrated in a graded ethanol series and sputter-coated with gold for 15-20 s. Micro-CT (Scanco Medical μ CT40) was used to image and characterize the mineral in the scaffold. Each scaffold ($n = 3$ per group per time point) was scanned at 16 μ m resolution (55 kVp, 172 mA, 200 ms) perpendicular to the longitudinal axis of the scaffold. The threshold was kept at 165 for all samples during the 6-week period. Using the manufacturer's software (Eval v5.0), the 3D structure of each scaffold was reconstructed and the volume information (bulk volume, total volume, and the ratio of bulk volume to total volume) was analyzed. To evaluate the distribution of bulk volume percentage in the scaffold, 8 positions of a $500 \times 500 \times 500 \mu\text{m}^3$ cube along the center line of the scaffold at the same Z-plane were chosen for analysis and the values were normalized to obtain the average bulk volume percentage of the scaffold.

Statistics

Experimental results were presented as means $\pm$ standard deviation (SD) for each scaffold. Statistical significance ($p < 0.05$) was determined by analysis of variance (ANOVA).

Results and Discussion

Fabrication of HAp-impregnated PLGA inverse opal scaffolds

Fig. 1 shows schematic diagrams and SEM images of each fabrication step for PLGA inverse opal scaffolds. Uniform gelatin microspheres with size of $225.8 \pm 2.4 \mu\text{m}$ were produced using a fluidic device.^{5a} This diameter was chosen based on the reported optimal range of pore sizes for bone regeneration ($100\text{--}250 \mu\text{m}$).^{3c} The gelatin microspheres dispersed in methanol were put into a 50-mL centrifuge tube and then crystallized into ccp lattice using an orbital shaker. To make the ccp lattice robust, the centrifuge tube containing the ccp lattice was carefully placed in an oven heated at 65°C for 1 h (Fig. 1A). The ccp lattice was placed in a filter paper to evaporate the solvent and then infiltrated with PLGA solution (20 wt%) in 1,4-dioxane. The ccp lattice with PLGA solution was then frozen in a refrigerator (-20°C) for 5 h and lyophilized in a freeze-dryer overnight (Fig. 1B). The gelatin ccp lattice with freeze-dried PLGA was immersed into warm water (45°C) with stirring for 3 h to remove gelatin microspheres (Fig. 1C).

Fig. 2 shows SEM images of the PLGA, PLGA/HAp, and Ap-coated PLGA/HAp scaffolds with a cylindrical shape (slightly conical, diameter at the top surface ≈ 4 mm, thickness ≈ 1.5 mm, and diameter at the base plane ≈ 5.5 mm). They exhibited remarkable uniformity and interconnectivity for pores throughout the scaffold. All the scaffolds contained uniform and spherical pores of $213 \mu\text{m}$ in diameter, which is slightly smaller than the diameter of the pristine gelatin microspheres. This difference is due to the shrinkage of the gelatin lattice during methanol evaporation. The PLGA/HAp scaffold displayed some HAp nanoparticles on the surface and the Ap-coated PLGA/HAp scaffold showed apatite crystals deposited on the surface (and thus increased surface roughness), whereas the PLGA scaffold showed a smooth surface. For the PLGA/HAp scaffolds, a mixture of PLGA and HAp nanoparticles in 1,4-dioxane was sonicated and infiltrated into the voids of the gelatin lattice. Furthermore, in order to decorate the scaffold surface and to improve the mechanical properties of the scaffold, bone-like apatite was deposited onto the surface of the PLGA/HAp scaffold using SBF.⁹ After incubation in $10\times$ SBF for different periods of time (Fig. 3), it was observed that

an increased amount of apatite was deposited on the surface of the scaffold. In this case, both the anionic groups of the PLGA and the HAp nanoparticles exposed on the surface of the scaffold could facilitate the nucleation process.¹⁰ The PLGA/HAp scaffolds showed higher compressive moduli than the PLGA scaffolds as already reported by many researchers:^{1b, 1c} 196.2 ± 23.8 kPa for the PLGA scaffolds, $1,744.7 \pm 68.6$ kPa for the PLGA/HAp scaffolds, $1,952.8 \pm 54.1$ kPa for Ap-coated PLGA/HAp scaffolds.

Culture of preosteoblast in the inverse opal scaffolds

MC3T3-E1 preosteoblasts were seeded onto the scaffold in a spinner flask and cell behavior was examined in response to osteogenic differentiation medium containing L-ascorbic acid and β -glycerol phosphate. As shown in Fig. 4, the cells initially proliferated in the PLGA scaffolds, but the proliferation rate slowed over time. In contrast, the cells in the Ap-coated PLGA/HAp scaffolds showed a nearly constant proliferation rate throughout the 4-week period. This may indicate that cells in the Ap-coated PLGA/HAp scaffolds were differentiating rather than proliferating. The cells in the PLGA/HAp scaffolds showed a proliferation behavior similar to that of the cells in the Ap-coated PLGA/HAp scaffolds. These results are consistent with a study performed by Shu *et al.*, who showed enhanced differentiation but decreased cell growth for osteoblasts seeded on HAp discs placed in differentiation media.¹¹ Fig. 5 shows SEM images of the cells in different scaffolds after 4 weeks of culture. Unlike the PLGA/HAp and Ap-coated PLGA/HAp scaffolds, most of the pores around the perimeter of the PLGA scaffolds were occluded by the cells and secreted ECM (left column in Fig. 5), presumably restricting the diffusion of culture media into the center of the scaffolds.¹² In addition, a large number of nodule-like mineral (indicated by arrows) were observed on the surface of the Ap-coated PLGA/HAp scaffold. In contrast, there were few mineral on the PLGA/HAp and PLGA scaffolds. These observations are likely related to the rough and stiff surfaces of the Ap-coated PLGA/HAp scaffolds, which provide favorable environments for the proliferation and differentiation of osteogenic cells.¹³

Micro-CT imaging and analysis

We employed micro-CT imaging to assess the amount and spatial distribution of secreted mineral inside the scaffolds. Fig. 6 shows representative micro-CT images of the cell/scaffold constructs at 1 day and 6 weeks post cell seeding. At 1 day, as shown in Fig. 6A, the PLGA scaffold was not visible when the standard threshold representing bone was used. In contrast, both the PLGA/HAp and Ap-coated PLGA/HAp scaffolds showed a well-arrayed uniform structure similar to the SEM images, indicating excellent uniformity in HAp distribution (Fig. 6, D and G). After 6 weeks of culture, the cross-sectional images of the PLGA/HAp and Ap-coated PLGA/HAp scaffolds confirmed the inverse opal structure developed inside the scaffold and also the uniform distribution of mineral secreted by the differentiated cells (Fig. 6, F and I). By contrast, the micro-CT images of the PLGA scaffolds showed only a small amount of mineral around the perimeter of the scaffolds (Fig. 6, B and C). This was likely because the cells crowded around the perimeter of the PLGA scaffolds exhausted the differentiation media, preventing differentiation of cells in the deeper regions of the scaffold. For bone tissue engineering, uniform mechanical strength throughout a scaffold is critical because a region with lower mechanical strength might easily fracture. In this regard, the HAp-impregnated scaffolds with an inverse opal structure may be preferable for bone regeneration due to their uniform distribution of HAp nanoparticles inside the scaffolds and also the uniform distribution of newly secreted mineral.

Despite the limitation of 16 μ m in resolution, micro-CT is a useful tool for assessing the cell/scaffold constructs because it can provide quantitative 3D information. The mineral

content of each cell/scaffold construct was calculated using a micro-CT volume analysis. The bulk volume percentage (BVP), corresponding to the mineral content, is defined as the percentage ratio of bulk volume (BV) relative to total volume (TV). Fig. 7A shows the changes over time in BVP for the PLGA, PLGA/HAp, and Ap-coated PLGA/HAp scaffolds, respectively. At 1 day, the HAp-impregnated scaffolds showed an average BVP value of about 23% (corresponding to 77% porosity), which is close to the void fraction of an inverse opal (26%). As expected, the PLGA scaffolds showed a BVP value of zero. In 6 weeks, the average BVP increased from 23.5 ± 2.4 to $36.2 \pm 3.7\%$ for the PLGA/HAp, and from 22.5 ± 2.3 to $47.4 \pm 2.7\%$ for the Ap-coated PLGA/HAp scaffolds, whereas the BVP for the PLGA scaffolds only increased from 0 to $1.2 \pm 0.5\%$. To microscopically evaluate the distribution of BVP within the scaffolds, we selected 8 cubes ($500 \times 500 \times 500 \mu\text{m}^3$) in the middle of the scaffold at the same Z-plane, and then calculated the BVP. As shown in Fig. 7B, the mineral secreted by the differentiated cells in the HAp-impregnated scaffold was evenly distributed throughout the scaffold, whereas the mineral was mainly deposited around the perimeter of the PLGA scaffold. These results suggest that the Ap-coated PLGA/HAp scaffold with an inverse opal structure could provide a favorable environment for preosteoblasts to differentiate and uniformly secrete a significant amount of mineral throughout the scaffold.

Conclusion

We have successfully fabricated inverse opal scaffolds composed of PLGA and HAp for bone tissue engineering. The incorporation of HAp nanoparticles and Ap-coating led to a 10-fold increase in the modulus of the scaffolds. After seeding preosteoblasts into the scaffolds (each with a different surface morphology and modulus) and adding osteogenic differentiation medium, the mineral content was measured using micro-CT volume analysis. It is worth noting that our microscopic evaluation of the mineral content in each cell/scaffold construct indicates that the secreted mineral was evenly distributed throughout the HAp-impregnated scaffolds. We showed that Ap-coated PLGA/HAp scaffolds provided the best environment among the three types of samples tested in terms of mechanical properties, osteoinductivity, and spatial distribution of mineral for bone tissue engineering. Taken together, we believe that the HAp-impregnated inverse opal scaffolds are promising for bone tissue engineering.

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Figure 1. Schematic diagrams and corresponding SEM images of (A) close cubic lattice of gelatin microspheres, (b) freeze-dried sample after infiltration of PLGA solution, and (C) PLGA inverse opal scaffold.

Figure 2. SEM images of (A, B) PLGA, (C, D) PLGA/HAp, and (E, F) Ap-coated PLGA/HAp scaffolds. The insets show magnified SEM images of the scaffold surface, where the scale bars are 2.5 μm .

Figure 3. SEM images showing the surfaces of the PLGA/HAp scaffolds in the course of Ap-coating using 10xSBF: (A) 30, (B) 60, (C) 90, and (D) 120 min.

Figure 4.
Proliferation of the preosteoblasts cultured on the different scaffolds ($n = 3$).

Figure 5. SEM images of the cells cultured on the (A) PLGA, (B) PLGA/HAp, and (C) Ap-coated PLGA/HAp scaffolds for 28 days. A large number of nodule-like mineral (indicated by arrows) were observed on the surface of the Ap-coated PLGA/HAp scaffolds.

Figure 6. Representative micro-CT images of (A-C) PLGA, (D-F) PLGA/HAp, and (G-I) Ap-coated PLGA/HAp scaffolds at one day and six weeks post cell seeding. (C, F, and I) Cross-sectional images taken at the 750 μ m plane in the Z-direction from the surface into the scaffold.

Figure 7.

(A) Plots of the bulk volume percentage (BVP) as a function of culture time for the PLGA, PLGA/HAp, Ap-coated PLGA/HAp scaffolds as determined by micro-CT volume analysis. The data are presented as mean $\pm$ SD ($n = 3$). Asterisk indicates significant difference ($p < 0.05$) relative to the value for the PLGA/HAp scaffold. (B) Plots of the normalized BVP values for the PLGA, PLGA/HAp, Ap-coated PLGA/HAp scaffolds along the center of the scaffold at the same Z-plane. All data were normalized to the average BVP of each scaffold.