
Engineering three-dimensional bone tissue *in vitro* using biodegradable scaffolds: Investigating initial cell-seeding density and culture period

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Abstract: New three-dimensional (3D) scaffolds for bone tissue engineering have been developed throughout which bone cells grow, differentiate, and produce mineralized matrix. In this study, the percentage of cells anchoring to our polymer scaffolds as a function of initial cell seeding density was established; we then investigated bone tissue formation throughout our scaffolds as a function of initial cell seeding density and time in culture. Initial cell seeding densities ranging from 0.5 to 10×10^6 cells/cm³ were seeded onto 3D scaffolds. After 1 h in culture, we determined that 25% of initial seeded cells had adhered to the scaffolds in static culture conditions. The cell-seeded scaffolds remained in culture for 3 and 6 weeks, to investigate the effect of initial cell seeding density on bone tissue formation *in vitro*. Further cultures using 1×10^6 cells/cm³ were maintained for 1 h and 1, 2, 4, and 6 weeks to study bone tissue formation as a function of culture period. After 3 and 6 weeks in culture,

scaffolds seeded with 1×10^6 cells/cm³ showed similar tissue formation as those seeded with higher initial cell seeding densities. When initial cell seeding densities of 1×10^6 cells/cm³ were used, osteocalcin immunolabeling indicative of osteoblast differentiation was seen throughout the scaffolds after only 2 weeks of culture. Von Kossa and tetracycline labeling, indicative of mineralization, occurred after 3 weeks. These results demonstrated that differentiated bone tissue was formed throughout 3D scaffolds after 2 weeks in culture using an optimized initial cell density, whereas mineralization of the tissue only occurred after 3 weeks. Furthermore, after 6 weeks in culture, newly formed bone tissue had replaced degrading polymer. © 2000 John Wiley & Sons, Inc. *J Biomed Mater Res*, 51, 376–382, 2000.

Key words: tissue engineering; bone; scaffolds; poly(lactide-co-glycolide)

INTRODUCTION

Bone injuries, genetic malformations, and diseases often require implantation of grafts. Three different grafting materials are currently used: autografts, allografts, and synthetic materials. Although autogenous bone is the “gold standard” graft material, it is limited in supply and necessitates traumatic harvesting procedures.¹ Allogeneous bone is inherently limited by risks of rejection and/or disease transmission, whereas permanent synthetic grafts are limited by osteolysis and inflammatory reactions to wear debris.^{2,3}

Tissue engineering is a new concept involving three-dimensional (3D) autologous tissue growth be-

fore implantation into patients. Ideally, bone tissue engineering will provide unlimited autologous bone tissue without the need of tissue harvesting procedures other than the relatively simple one of harvesting marrow cells from the patient.

Scaffolds for tissue engineering have been fabricated using biodegradable, biocompatible, and noncytotoxic polymers; in particular, poly(lactide-co-glycolide) (PLGA) 75/25 has been molded into porous structures that serve as scaffolds for bone marrow-derived cells.^{4,5} Bone marrow cells grown on these structures produced an outer, superficial mineralized tissue with little to no bone tissue within the scaffolds.^{6,7} Although these scaffolds demonstrated the promise of bone tissue engineering, their overall geometry was inappropriate for cell infiltration and 3D tissue formation.

To obtain bone cell colonization and bone tissue formation throughout entire scaffolds, we prepared new scaffolds with a morphology similar to that of trabec-

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ular bone, having open-cell pores with a high degree of interconnectivity.⁸ In culture, bone marrow-derived cells infiltrated and produced new bone tissue throughout the entire 3D volume of the scaffolds.⁹

To gain further insight into bone tissue formation throughout our 3D scaffolds, we investigated the optimal cell seeding density and culture period for bone tissue formation *in vitro* using femoral rodent bone marrow-derived cells. We used an osteocalcin immunolabeling assay to demonstrate the osteoblastic phenotype of the cells growing within the 3D scaffolds and tetracycline and von Kossa labeling to show mineralized tissue formation at different culture time points.

Histological sections were analyzed for new tissue formation on our 3D scaffolds. The morphology of cells growing throughout the scaffolds was visualized by analyzing foam sections maintained in culture for 1 h and 1 week, whereas the morphology and mineral content of newly formed tissue were observed histologically after 2, 4, and 6 weeks. Thus we investigated cell growth, differentiation, and matrix formation throughout the scaffolds.

MATERIALS AND METHODS

Deionized distilled water (ddH₂O) was obtained from Milli-RO 10 Plus and Milli-Q UF Plus apparatus (Bedford, MA) and used at 18 mΩ resistance. PLGA 75/25 (Birmingham Polymers, Birmingham, AL) had an intrinsic viscosity of 0.87 dL/g at 30°C in chloroform. The weight average molecular weight (M_w) of the polymer was determined by gel permeation chromatography (GPC), relative to polystyrene standards, to be 81,500 g/mol. Samples were sputter-coated with gold (approximately 10 nm) (Polaron Instrument, Doylestown PA) before examination by scanning electron microscopy (SEM). SEM micrographs were taken on a Hitachi 2500 at 15 kV acceleration voltage. An Ames Lab-tek cryostat (Elkhart, IN) was used at -20°C; histological sections were examined by light microscopy (LM; Leitz, Heerbrugg, Switzerland) and pictures were taken on 100 ASA Kodak film.

Scaffold formation

The PLGA 75/25 scaffolds were prepared as previously described.⁸ Briefly, glucose crystals were dispersed in a PLGA 75/25 solution in dimethylsulfoxide (DMSO; BDH, Toronto, Ontario, Canada). The polymer was precipitated and the glucose crystals were extracted in water from the precipitated polymer. Scaffolds were dried to constant mass (0.01 mm Hg, 72 h) before use. The scaffold dimensions were approximately 5 × 7 × 7 mm³.

Bone marrow-derived cell culture

First-passage primary bone marrow-derived cells were seeded on scaffolds using protocols and media described in

detail elsewhere.¹⁰ Briefly, bone marrow-derived cells were collected from both femora of young adult male Wistar rats (~150 g) into a fully supplemented medium (FSM): α-minimal essential medium (α-MEM) supplemented with 15% fetal bovine serum, 50 mg/mL ascorbic acid, 10 mM β-glycerophosphate, antibiotics (0.1 mg/mL penicillin G, 0.05 mg/mL gentamicin, and 0.3 mg/mL fungizone), and 10⁻⁸M dexamethasone (Dex). Control fibroblastic cultures were cultured in Dex-free FSM.¹¹

Cells were maintained in culture for 6 days and refed at days 2 and 5 with FSM. At day 6, cells were trypsinized with 0.01% trypsin and 10 μM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Preliminary studies were conducted to determine the seeding efficacy on the scaffolds in static culture conditions. Cells were seeded onto prewetted scaffolds at concentrations of 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 × 10⁶ cells/cm³. Because the scaffolds presented slight variations in size, the number of cells seeded onto the scaffolds was normalized to scaffold volume. After 1 h, cells on the culture dish were removed, counted, and expressed as a percentage of the initial cell density. The cell-seeded scaffolds were further used to study the effect of initial cell-seeding density on bone tissue formation *in vitro*: Three scaffolds seeded with all initial cell seeding concentrations from 0.5 to 6.0 × 10⁶ cells/cm³, as described above, were maintained in culture for 3 and 6 weeks and refed with FSM every 2–3 days. To study the effect of culture periods on bone tissue formation, 15 scaffolds were seeded with 1 × 10⁶ cells/cm³. These structures were maintained in culture for the following periods: 1 h and 1, 2, 4, and 6 weeks.

A new tetracycline-containing fully supplemented medium (TFSM) was prepared of α-MEM containing 15% fetal bovine serum, 50 mg/mL ascorbic acid, 10 mM β-glycerophosphate, and 9 mg/mL tetracycline·HCl (Sigma, St. Louis, MO). The TFSM was used for the last refeeding of all cultures. Cultures were then washed in α-MEM and fixed overnight in Karnovsky's fixative (2.0% paraformaldehyde, 2.5% glutaraldehyde, and 0.1M sodium cacodylate buffer, pH 7.2–7.4).

Imaging of tetracycline fluorescence

Entire cultured scaffolds were photographed under ultraviolet (UV) light with a Nikon camera (F-601 equipped with AF Micro Nikon 60-mm lens) using color slide film (Ektachrome, 400 ASA). A custom-built box contained the UV irradiation and limited light from external sources. The UV source consisted of four lamps (Microlites Scientific, Ontario, Canada; 365-nm wavelength) positioned circumferentially inside the custom-built box. A UV filter and a broad-band interference filter (λ = 550 nm; Melles Griot, Irvine, CA) was fitted to the lens to narrow the band of transmitted light to the 500- to 600-nm range; the emitted fluorescence of tetracycline was in the 530-nm range.

Histological preparation

Histology was done as previously described.¹² Briefly, fixed cultures on scaffolds were stained with hematoxylin

and eosin (H&E) *in situ*, embedded in Tissue-Tek, and sectioned at a thickness of 10 μm . Scaffold sections were further counterstained with von Kossa silver nitrate stain for visualization of mineralized matrix.

Determination of osteocalcin expression

Fixed cultures on scaffolds were immunolabeled *in situ* using a goat anti-rat osteocalcin antiserum (Biomedical Technologies, Stoughton MA) at a final dilution of 1:6000. The assay was terminated by second antibody labeling with donkey anti-goat immunoglobulin G conjugated to horseradish peroxidase antiserum, at a concentration of 1:250. A DAB substrate kit for peroxidase (Vector Laboratories, Burlingame, CA) was used supplemented with nickel chloride to develop the staining.

RESULTS

Biodegradable PLGA 75/25 scaffolds exhibited a regular distribution of interconnected macropores separated from each other by struts of microporous polymer and similar in overall morphology to that of trabecular bone. As shown in Figure 1(a,b), the macropores ranged in diameter from 1.5 to 2.2 mm, whereas polymer struts measured between 100 and 200 μm in thickness and had several micropores throughout.

Effect of cell seeding density and culture period on cell differentiation and bone tissue formation

While the initial cell seeding concentration was varied from 0.5 to 10×10^6 cells/ cm^3 , a maximum of 25%

of the initial cell seeding concentration remained on the foams. In fact, a plateau of 1.5×10^6 cells/ cm^3 was reached regardless of the initial cell seeding concentration. The other 75% of cells was found at the bottom of the tissue culture dish, and thus did not contribute to new tissue formation on the scaffold. This limited cell seeding may be attributed to the static culture system used and would likely improve in a dynamic system.

Notwithstanding the inefficient cell seeding methodology used, we had strong evidence supporting cell differentiation and mineralized tissue formation throughout the entire scaffold. As summarized in Table I, cells seeded at different initial seeding densities were investigated after 3 and 6 weeks in culture for both mineralized matrix formation by tetracycline staining and osteocalcin expression by immunolabeling. Dex was added to the media to induce proliferation and differentiation of osteogenic precursor cells, whereas control Dex-free media allowed the proliferation of mostly fibroblastic cells. After 3 weeks in culture, only those scaffolds seeded with 4×10^6 cells/ cm^3 or more showed tetracycline-labeled foci within their 3D volume. After 6 weeks in culture, however, all scaffolds seeded with 1×10^6 cells/ cm^3 or more showed fluorescent foci within their 3D volume. Figure 2 illustrates tetracycline staining on scaffolds after 6 weeks culture in (a) Dex-supplemented media and (b) control Dex-free media. Whereas scaffolds cultured in Dex-supplemented media clearly demonstrated strong tetracycline labeling indicative of mineralized matrix elaboration, those maintained in Dex-free media showed only faint background labeling.

After 3 weeks in culture, all scaffolds showed im-

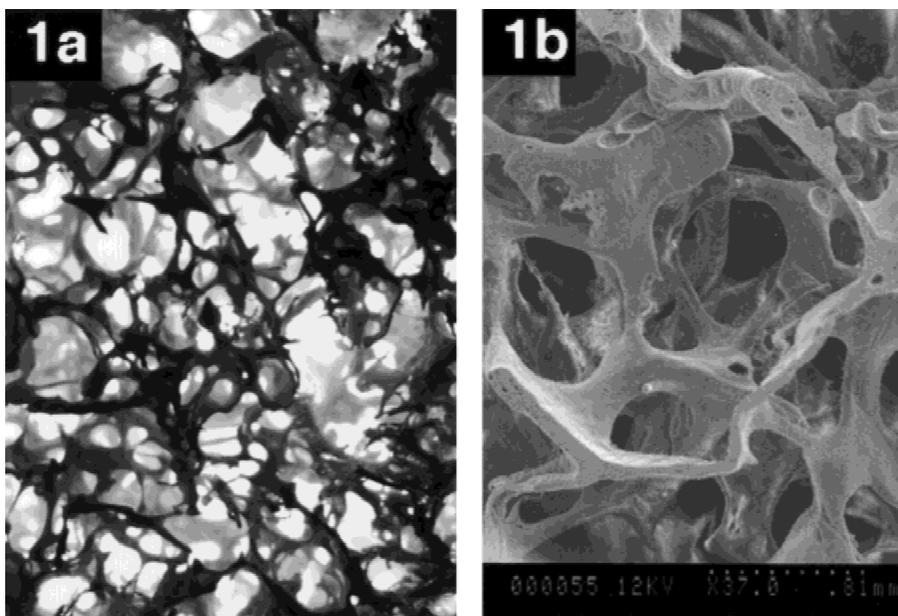


Figure 1. (a) Light micrograph of a PLGA 75/25 scaffold (field width = 0.6 cm); (b) scanning electron micrograph of a PLGA 75/25 scaffold.

TABLE I
Effect of Initial Cell Seeding Density on Cell Differentiation and Matrix Elaboration

Cell Seeding Density ($\times 10^{-6}/\text{cm}^3$)	Week 3		Week 6	
	Osteocalcin	Tetracycline	Osteocalcin	Tetracycline
0.5	Surface labeling only	Scattered fluorescent foci on scaffold surface	Strong surface labeling, weak labeling at center of scaffold	Scattered fluorescent foci on scaffold surface, few fluorescent foci within scaffold
1.0	Strong surface labeling, weak labeling at center of scaffold	Scattered fluorescent foci on scaffold surface	Strong labeling throughout entire scaffold	Fluorescent foci throughout scaffold
2.0	Strong surface labeling, weak labeling at center of scaffold	Scattered fluorescent foci on scaffold surface	Strong labeling throughout entire scaffold	Fluorescent foci throughout scaffold
3.0	Strong surface labeling, weak labeling at center of scaffold	Scattered fluorescent foci on scaffold surface	Strong labeling throughout entire scaffold	Fluorescent foci throughout scaffold
4.0	Strong surface labeling, weak labeling at center of scaffold	Fluorescent foci on edges and center of scaffold	Strong labeling throughout entire scaffold	Fluorescent foci throughout scaffold
6.0	Strong labeling throughout entire scaffold	Fluorescent foci throughout scaffold	Strong labeling throughout entire scaffold	Fluorescent foci throughout scaffold

munolabeling for osteocalcin. Strong labeling was evident throughout the entire volume of scaffolds seeded with an initial 6.0×10^6 cells/cm³; strong labeling was observed on the outer edges, with only weak labeling in the center of all other cell concentration seeded on scaffolds. After 6 weeks in culture, all scaffolds showed equal immunolabeling for osteocalcin throughout their entire volume except those seeded at the lowest initial concentration studied, 0.5×10^6 cells/cm³, where only surface immunolabeling was strong. Figure 3 illustrates osteocalcin labeling on (a) Dex-supplemented cultured scaffolds after 6 weeks in culture, and (b) Dex-free control cultured scaffolds. Figure 3(c) illustrates a control Dex-supplemented culture not incubated in the anti-osteocalcin antibody, but submitted to all other steps of immunolabeling. No background immunolabeling was found on either control, confirming the importance of Dex for osteoblast differentiation and the specificity of the osteocalcin antibody.

Histological observations

Scaffolds maintained in culture for 1 h and 1, 2, 4, and 6 weeks with an initial cell seeding density of 1×10^6 cells/cm³ were stained with H&E, sectioned, and counterstained with von Kossa, as shown in Figure 4(a–e). After only 1 h, cells were found throughout the 3D volume of the scaffolds. Whereas most cells exhibited a rounded morphology, there was some evidence of cell spreading at this early time [Fig. 4(a)]. After 1 week in culture, monolayers of cells were found lining the pore walls; however, much of the pore wall surface

was left uncovered [Fig. 4(b)]. As culture time progressed, cell spreading and tissue formation increased. After 2 weeks in culture, most of the pore walls were covered with more than one cell layer throughout the entire volume of the scaffolds [Fig. 4(c)]. A few von Kossa-stained areas indicative of mineralized tissue were found on the outer edges of the scaffolds. After 4 weeks, a dense layer of tissue continuously counter-

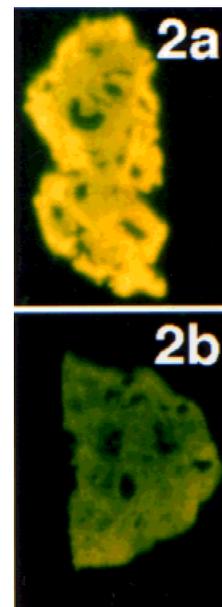


Figure 2. Tetracycline staining after 6 weeks in culture on (a) a scaffold cultured in Dex-supplemented media, and (b) a control scaffold cultured in Dex-free media. Field widths of = 1.0 cm. Bright yellow fluorescence was observed throughout the entire scaffold of (a), whereas fibroblastic control cultured foams only showed background staining (b).

stained with von Kossa was found throughout the volume of the scaffold [Fig. 4(d)]. Thicker tissue with strong von Kossa counterstaining was observed throughout the entire scaffold volume after 6 weeks in culture. Evidence that degrading polymer struts were being infiltrated with cells and new tissue indicated that the bulk polymer would ultimately be replaced with tissue [Fig. 4(e)]. These data with tetracycline and osteocalcin immunolabeling are summarized in Table II for effect of culture period on cell differentiation and matrix formation.

DISCUSSION

We studied the effects of different initial cell seeding densities on tissue formation after 3- and 6-week culture periods in terms of cell differentiation (osteocalcin immunolabeling) and mineralized matrix formation (von Kossa staining and tetracycline labeling).

After 3 weeks in culture, all initial cell seeding densities resulted in surface immunolabeling. With cell seeding densities of $1.0\text{--}4.0 \times 10^6$ cells/cm³, labeling was found throughout the entire foam structure, albeit weak. Higher initial seeding densities (6.0×10^6 cells/cm³) showed strong labeling throughout the foams. After 6 weeks in culture, all scaffolds except those seeded with 0.5×10^6 cells/cm³ were equally labeled throughout their 3D volume. The differences observed in tissue formation at 3 and 6 weeks indicated that multilayering and differentiation of cells occurred earlier in the outer edges than in the middle areas of the scaffolds. Thus, cell differentiation occurred at different rates throughout the scaffolds, likely due to an uneven distribution of cells within the scaffolds during the initial cell-seeding procedure. Initially, more cells likely anchored to the outer edges than in the center of the scaffolds.

Tetracycline staining confirmed a similar time lapse of mineralized tissue formation, with that on the outer edge occurring before that in the center of the scaffold.

For all cell densities investigated except 6.0×10^6 cells/cm³, both tetracycline labeling and von Kossa staining were visible at the edges before the center of the scaffolds. Those scaffolds seeded with 6×10^6 cells/cm³ had an even distribution of tetracycline and von Kossa-stained tissue. Importantly, the mineralization on the outer surface did not preclude mineralization or bone formation throughout the entire foam structure as observed at later times.

After six weeks in culture, tissue formation was independent of initial cell-seeding density, whether it was 1.0×10^6 or 6.0×10^6 cells/cm³. Thus, a cell density of 1.0×10^6 cells/cm³ was used to investigate the effect of culture period on tissue formation *in vitro*. Tissue formation after different culture periods was observed histologically and characterized by osteocalcin immunolabeling and tetracycline staining. After 1 h in culture, cells were found throughout the entire scaffold, indicating that tissue formation within the bulk of the polymer foam did not rely on the migration of the cells from the surface of the foam structure toward its center. In fact, rounded cells, as shown in Figure 4(a), were observed throughout the entire scaffold. Von Kossa- and tetracycline-stained tissues were observed after 3 weeks in culture, whereas osteocalcin labeling was observed after 2 weeks in culture. This confirms that matrix mineralization was preceded by osteocalcin expression, as previously observed.¹³ (In the current study, histomorphometry was not conducted because the goal of this investigation was to determine the optimal cell culture conditions required for bone tissue formation throughout 3D scaffolds *in vitro*, and not the amount of mineralized bone matrix obtained for each cell seeding density.)

After 6 weeks in culture, some of the polymer struts had degraded into smaller fragments, as shown in Figure 4(e). Importantly for tissue engineering, new tissue invaded the space previously occupied by the polymer. Whereas previous studies indicated the potential detrimental side effects of PLGA for tissue en-

TABLE II
Effect of Culture Period of Cell Differentiation and Matrix Formation *In Vitro*

Time in Culture	Cell Morphology	Osteocalcin	von Kossa	Tetracycline
1 h	Rounded		—	—
2 wk	Spread	Faint throughout, brighter on specific spots on outer part of foams	Scattered staining on edges of scaffold	—
3 wk	Spread	Strong surface labeling, weak labeling at center of scaffold	Scattered staining on edges of scaffold	Scattered fluorescent foci on scaffold surface
4 wk	Spread	Strong labeling throughout entire scaffold	Staining throughout, with greater staining on outer surface of scaffolds	Fluorescent foci throughout scaffold, with brighter surface fluorescent foci
6 wk	Spread	Strong labeling throughout entire scaffold	Strong labeling throughout entire scaffold	Strong labeling throughout entire scaffold

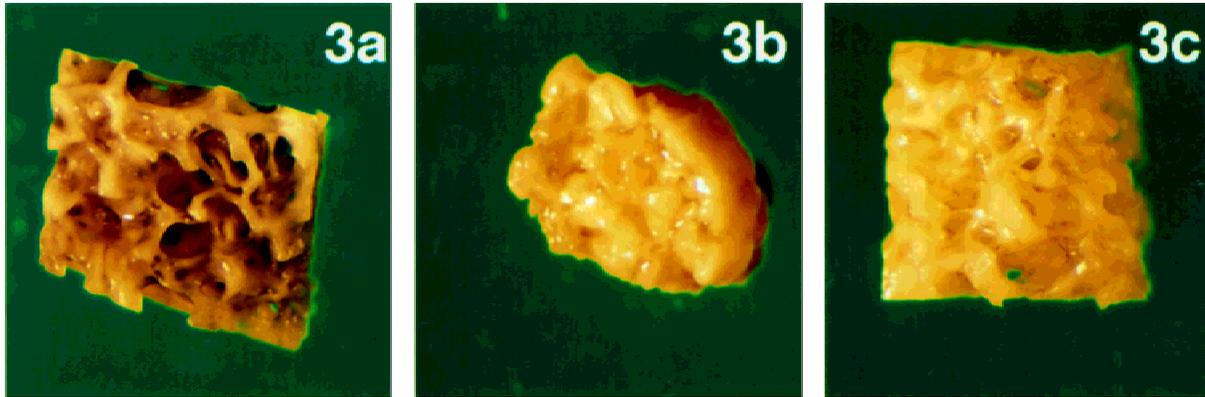


Figure 3. Light micrographs of osteocalcin-immunolabeled cultures after 6 weeks in culture. (a) Scaffolds cultured in Dex-supplemented media showed strong immunolabeling on the pore walls and within the pore wall structures; (b) control fibroblastic cultures and (c) control scaffold cultured in Dex-supplemented media, but not incubated with the osteocalcin antibody, showed no background stain. Field widths = 0.6 cm.

gineering applications,¹⁴ these results demonstrate that the degraded PLGA is innocuous to cells, allowing them to fill areas previously occupied by polymer with tissue. Other authors showed that the acidic milieu associated with PLGA degradation is toxic to cells, but we had no evidence of cytotoxicity. We attribute this difference to the highly porous open cell foam structure of our PLGA scaffolds. It is important

to note that our scaffolds are mostly fluid-filled, allowing for the facile diffusion of degradation products. The histology shown here demonstrates that cellular action on these polymer scaffolds accelerates polymer degradation; previous degradation studies showed that these scaffolds were stable *in vitro* for at least 18 weeks at pH 6.4 and 7.4 and at least 16 weeks at pH 5.0.¹⁵ In these studies, we had our first evidence

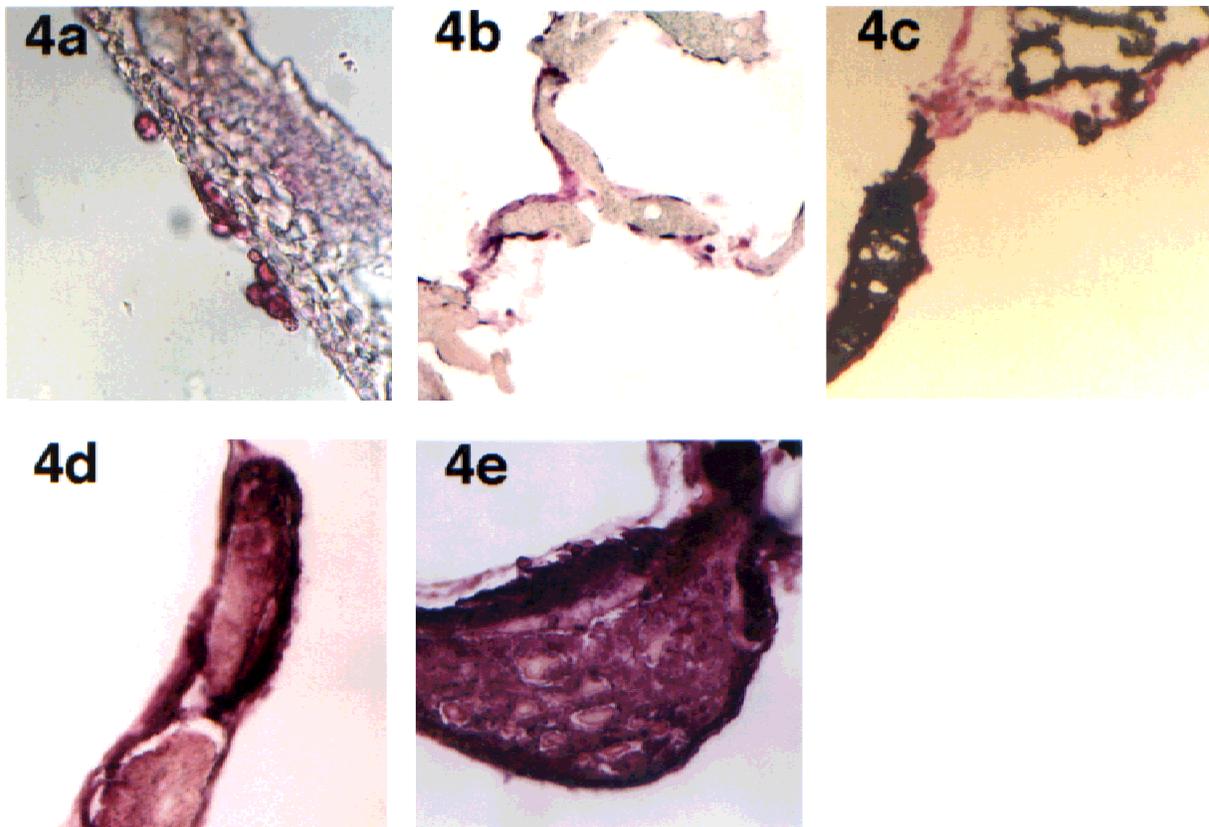


Figure 4. Histological sections of scaffold structures stained with H&E and counterstained with von Kossa after the following times in culture: (a) 1 h (field width = 39 μm), (b) 1 week (field width = 365 μm), (c) 2 weeks (field width = 365 μm), (d) 4 weeks (field width = 145 μm), and (e) 6 weeks (field width = 145 μm).

of degradation at 6 weeks, with a change in scaffold morphology.

CONCLUSIONS

We have shown that new bone forms throughout 3D scaffolds primarily as a function of culture period. The initial cell seeding density did not significantly affect tissue formation, and lowering seeding densities only delayed the formation of mineralized tissue. Interestingly, as the scaffold degraded it was replaced with new tissue. This is critical to the success of bone tissue engineering on biodegradable polymeric scaffolds, as the premise relies on newly engineered tissue replacing the scaffold over time. We demonstrated in this study that the degrading polymer scaffold indeed began to be replaced by new mineralized tissue after a 6-week culture period. Future studies include the implantation of these tissue-engineered products *in vivo*.

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