Bone formation on two-dimensional poly(DL-lactide-*co*-glycolide) (PLGA) films and three-dimensional PLGA tissue engineering scaffolds *in vitro*

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Abstract: For some bone tissue engineering strategies, direct contact of newly synthesized bone with a scaffold is important for structural continuity and stability at the scaffold/bone interface. Thus, as the polymer degrades, the support function of the scaffold could be adopted by the developing bone structure. This study was designed to determine whether poly(DL-lactide-*co*-glycolide) with a comonomer ratio of 75:25 supports bone apposition *in vitro*. Osteogenic cells derived from rat bone marrow cells were cultured for 2 weeks on polymeric two-dimensional films and three-dimensional tissue engineering scaffolds. Bacteriological grade polystyrene and tissue culture polystyrene dishes served as negative and positive controls for interfacial bone deposition, respectively. The surfaces of the prepared substrates were characterized by X-ray photoelectron spectroscopy, dynamic water contact angle, scanning electron microscopy, and atomic force microscopy. After cell culture, the elaborated matrix was examined using scanning electron microscopy, transmission electron microscopy, and energy dispersive spectroscopy. The results show that poly(DL-lactide-*co*-glycolide) supports appositional bone growth on both two-dimensional films and three-dimensional scaffolds, including the formation of a mineralized cement line matrix. Furthermore, surface topographical features are not required for the adherence of the cement line matrix to the polymer. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 64A: 388–396, 2003

Key words: bone tissue engineering; scaffold; trabecular bone; cement line matrix; biomaterial interface

INTRODUCTION

Poly(DL-lactide-*co*-glycolide) (PLGA) is a biodegradable polymer that has been studied extensively since the 1960s and is commonly used as suture material[1] and in drug-delivery devices.[2] Bone implants are also made of PLGA and have been shown to accelerate bone healing or induce more rapid bone formation in comparison to untreated controls.[3] Although some inflammatory responses have been reported, PLGA has proven useful for bone applications, especially when the material volume is relatively small.[4] Indeed, scaffolds made from these materials offer great potential for bone tissue-engineering applications, and bone has been shown to grow, within such scaffolds, both *in vitro* and *in vivo*.[5–8] However, despite numerous studies, none have demonstrated direct contact of bone matrix with the polymer surface.

It has now been well documented that when bone matrix is formed *de novo* at solid surfaces, the bone/substrate interface is occupied by a calcified interfacial matrix that is elaborated by differentiating osteogenic cells and histologically identified as the cement line.[9] Cement lines are seen in naturally remodeling bone as boundary lines between secondary osteonal and surrounding interstitial bone.[10] They comprise a thin layer (~0.5 μm) of mineralized collagen-free matrix, which serves as a scaffold for the assembly of the developing collagenous matrix and connects new bone to the old bone surface. It is considered that because of their organic and mineral composition, which is different from the bulk bone matrix, cement lines have

reduced stiffness and static strength. This enables them to absorb energy and provides the resistance of osteonal bone to fatigue damage and crack growth.[11,12] Thus, the cement line is an interfacial structure in bone tissue, which provides many biomechanical characteristics in addition to adhesive functions.[13,14]

Cement lines have been observed on a variety of biomaterial surfaces both *in vitro* and *in vivo* since their original experimental demonstration in 1991;[15] however, they have not been observed on PLGA. Since PLGA releases acidic degradation products, these could compromise the calcium hydroxyapatite component of the cement line interface and thus the bone/polymer interface itself. Our goal was to determine whether a cement line matrix could be deposited directly onto a PLGA surface and whether this matrix remained mineralized *in vitro*. To answer these questions, we focused on two-dimensional (2D) PLGA model film surfaces, as this facilitated analysis, and then translated these findings to three-dimensional (3D) PLGA scaffolds that may find utility *in vivo*.

MATERIALS AND METHODS

All materials were used as received unless otherwise indicated. The following substrates were used: bacteriological grade polystyrene (BgPS; 35 mm^2, Falcon®), tissue culture polystyrene (TCPS; 35 mm^2, Corning Glass Works, NY), glass coverslips (2.54 cm^2, Bellco, NJ), Teflon® PFA, (VWR Canlab, Mississauga, Ontario, Canada) and tissue culture flasks (75 cm^2, Falcon®). PLGA 75:25, having an inherent viscosity of 0.87dL/g at 30°C in chloroform, was obtained from Birmingham Polymers Inc. (Birmingham, AL). Chloroform was obtained from Caledon Laboratories Ltd. Dimethysulfoxide was obtained from BDH Inc (Toronto, Ontario). The α-minimal essential medium (α-MEM), fetal calf serum, trypsin, and gentamicin were obtained from GIBCO BRL. Nylon cell strainers (70 μm) were purchased from Becton Dickinson Labware, (Lincoln Park, NJ). The penicillin G, amphotericin B, L-ascorbic acid, sodium-β-glycerophosphate, and dexamethasone were obtained from Sigma Chemical Company (St. Louis, MO). Deionized distilled water was obtained from a Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) and used at 18MΩ resistance.

Cell isolation

Rat bone marrow cells were obtained according to previously published methods.[16] Briefly, femora were excised from young adult (115–125 g) Wistar rats (Charles River) and transferred into an antibiotic/fungizone solution consisting of penicillin G (1670 units/mL), gentamicin (500 μg/mL), amphotericin B (3 μg/mL). After 10 min, femora were transferred into a fresh aliquot of the same solution. This washing procedure was repeated three times. Subsequently, the femora were transferred into α-MEM. Epiphyses were removed, and the marrow from each diaphysis was flushed out with 15 mL of α-MEM supplemented with 15% (v/v) fetal calf serum, 100 mg of L-ascorbic acid, 5 mM β-glycerophosphate, and 10^{-8} M dexamethasone, together with antibiotics and fungizone at one-tenth the concentration used during excision. Marrow cells of both diaphyses were collected and cultured in the supplemented α-MEM.

Polymer-coated glass coverslips

PLGA 75:25 was dissolved in chloroform at 2% (w/v). Glass coverslips were sterilized at 200°C in a ceramic furnace (Lindberg, Asheville, NC) for 2 h. A photolithographic spinner (Headway Research Inc., TX) was used to spin coat sterile glass coverslips using 0.5 mL of a 2% w/v PLGA-chloroform solution (120 s at 5500 rpm). The coverslips were then air-dried, disinfected in 70% ethanol for 30 min, and rinsed five times with α-MEM before seeding with cells. The films were <100 nm in thickness, as determined from scanning electron microscopy (SEM) images. Non-coated glass coverslips (controls for spin-coating) were treated identically to polymer-coated coverslips. Sterile TCPS served as a control for cell culture.

PLGA films cast onto Teflon® dishes

PLGA 75:25 was dissolved in chloroform at 8% (w/v), and 20 mL was cast into 50-mm diameter Teflon® dishes. Films were air-dried for 24 h and then dried under vacuum for 2 h at room temperature. The films were ~400-μm thick, as measured with digimatic calipers. Polymer films were disinfected in 70% ethanol for 30 min, rinsed five times with α-MEM, and then incubated for 24 h in α-MEM before seeding with cells. PLGA films were left in the Teflon® dishes for the duration of the culture experiments.

3D PLGA scaffolds

PLGA 75:25 3-D scaffolds were prepared as previously described.[17] Briefly, glucose crystals were dispersed in a 0.1g/mL PLGA 75:25 solution in dimethysulfoxide, and this was placed at −15°C for 30 min in a 10 × 10-cm aluminum foil, 3-cm deep mold. The polymer was precipitated, and the glucose crystals were extracted from the precipitated polymer by placing the frozen scaffold block into water. Scaffolds were prepared to have a pore size of ~1.5 mm in diameter, a porosity ~ 92%, and a pore wall thickness ~200 μm, as described by Holy.[18] Scaffolds were rinsed thoroughly with deionized distilled water. Foams were cut by hand into cubes measuring 0.6 × 0.6 × 0.6 cm^3 and disinfected in 70% ethanol for 30 min, rinsed five times with α-MEM, and then incubated for 24 h in α-MEM before seeding with cells.

Cell culture

Fifteen milliliters of the cell suspension were cultured in T75 flasks maintained at 37°C in an incubator with an atmosphere consisting of 95% air, 5% CO_2, and 100% relative humidity. The medium was changed on days 1 and 4. On day 5 or 6, depending on cell confluency, the cells were subcultured by enzymatic release using 0.01% trypsin. An aliquot of the trypsinized cells was passed through a cell strainer and then counted using a Coulter Counter® (model ZM Coulter Electronics Ltd., Luton, Beds, UK). Cells were seeded on 70% ethanol disinfected PLGA-coated glass coverslips and 3D scaffolds at a concentration of 10^4cells/cm^2 and 10^6cells/cm^3, respectively. Cells were dispensed dropwise on 3D scaffolds, contained in the individual wells of a 24-well culture plate, using a 10-mL syringe fitted with a 20-gauge needle. Cultures were maintained for 14 days and refed every 2 to 3 days. Polymer-coated coverslips and 3D scaffolds were immersed in the tissue culture medium but not seeded with cells to serve as controls for polymer degradation.

Surface characterization

2D surfaces were characterized by 1) dynamic advancing and receding water contact angles to assess the relative hydrophobicity of the various substrates; 2) X-ray photoelectron spectroscopy (XPS) to determine surface elemental composition; and 3) SEM to observe surface topography. PLGA spin-coated glass coverslips were also characterized by atom force microscopy (AFM) to examine surface topography. 3D PLGA scaffolds were characterized by SEM to examine surface morphology and topography. The porosity and degree of interconnectedness of the macropores were previously determined.[18] Transmission electron microscopy (TEM) was used to examine the bone-PLGA interface for cultures grown on PLGA films that were cast in Teflon® dishes.

Advancing and receding water contact angles were obtained on a Rame-Hart NRL telescopic goniometer (Mountain Lakes, NJ). Values reported represent the average and standard deviation of five measurements taken from each of three samples of each surface for a total of 15 measurements.

The elemental composition of the surface of the polymer substrates was analyzed using XPS with a 90-degree take-off angle. The spectrum was obtained on a Leybold MAX 200 XPS system utilizing an unmonochromatized MgKα X-ray source operating at 12 kV and 25 mA. Values reported represent the average of one measurement taken from each of three samples of each surface. All standard deviations for XPS data were less than ± 3.3%.

After processing, polymer films were dried under a vacuum at room temperature for 2 h, placed into a desiccator for 24 h, and then analyzed by AFM. All data were acquired in air using a Digital Instruments Nanoscope IIIA Multimode scanning probe microscope in tapping mode. The samples were mounted on steel pucks using double-sided adhesive tape and mounted on an E-scanner, which has a maximum scan size of 13.5 μm × 13.5 μm. All images were acquired using OTESPA silicon diving board cantilevers with integral bi-pyramidal tips at a tip oscillation frequency of ~250 kHz and an image scan rate of ~2 Hz as 512 × 512 pixel data sets. The feedback conditions (gains/drive and set-point amplitudes) were adjusted to optimize image quality.

Before fixation, culture substrates were washed three times in serum-free α-MEM at 37°C and then twice with 0.1M Na-cacodylate buffer (pH 7.4, 37°C). Fixation was carried out for a minimum of 2 h in Karnovsky's fixative at 4°C. After rinsing with cacodylate buffer three times, the dishes were dehydrated in graded alcohols (50%, 70%, 80%, 90%, 95%, and 100%) and then critical point dried from CO_2 (Ladd Research Industries Inc., Burlington, VT). Overlying cell layers and the collagenous matrix were partially removed with compressed air to facilitate examination of the elaborated mineralized extracellular matrix. The samples were then sputter-coated with platinum (~10 nm; Polaron Instrument Inc., Doylestown, PA) and examined in an Hitachi (model 2500) scanning electron microscope at 10 kV.

Films cast onto Teflon® petri dishes were fixed with Karnovsky's fixative and post fixed with osmium tetroxide. All samples were thoroughly washed before enbloc staining with uranyl acetate in 50% ethanol for 2 h at room temperature. Specimens were dehydrated in graded alcohols and infiltrated with a series of alcohol-epon mixtures and finally with two changes of pure epon. The epon was polymerized at 40°C overnight and then at 60°C for an additional 3 days. Selected areas of the tissue were re-embedded in beam capsules. Silver to pale gold thin sections were cut on an ultramicrotome and mounted on copper grids. The sections were stained with 3% uranyl acetate in 30% ethanol and Reynold's lead citrate and examined in a Phillips 400T transmission electron microscope equipped with a Phillips EM430 energy dispersive spectroscopy (EDS) and a Link analytical AN10000 X-ray analyzer.

RESULTS

Surface analysis

PLGA-coated glass coverslips, TCPS dishes, BgPS dishes, PLGA-coated Teflon® dishes, and glass coverslips were characterized by XPS and by dynamic advancing (θ_A) and receding (θ_R) water contact angles. The XPS data and contact angle data are summarized in Table I. Glass coverslips were coated with PLGA, as evidenced by the absence of the silicon XPS peak and increased contact angles relative to glass (see Table I). PLGA-coated glass coverslips and PLGA-coated Teflon® petri dishes exhibited similar elemental surface compositions and hydrophobicity, reflecting similar surface properties regardless of casting surface. TCPS and BgPS exhibited the same elemental surface composition yet different contact angles, reflecting different surface energies. BgPS and PLGA had similar receding contact angles yet different advancing contact

TABLE I
XPS Elemental Composition Results at a 90-degree Takeoff Angle ($n = 3$) and Dynamic Water Contact Angle Results ($n = 15$, mean ± standard deviation)

Material Surface	C[a]	O	Si	Contact Angle θ_A[b]$/\theta_R$ ($n = 15$)
PLGA (coated glass coverslips)	61%[c]	39%	0%	71° ± 2°/51° ± 2°
PLGA (cast on Teflon® dishes)	60%	40%	0%	73° ± 3°/52° ± 4°
Glass	15%	62%	24%	61° ± 2°/34° ± 1°
Tissue culture polystyrene	83%	17%	0%	61° ± 3°/16° ± 2°
Bacteriological-grade polystyrene	83%	17%	0%	88° ± 2°/47° ± 2°

[a]C, carbon; O, oxygen; Si, silicon.
[b]θ_A, advancing water contact angle; θ_R, receding water contact angle.
[c]All standard deviations for XPS data were less than ±3.3%.

angles, indicating that BgPS was likely more hydrophobic than PLGA.

PLGA-coated glass coverslips were further characterized by AFM to determine the surface topography of the coating (Fig. 1). The surfaces appeared smooth, having few distinguishing features with topographical effects <5 nm in height and having an average surface roughness of under 1 nm. Furthermore, PLGA-coated glass coverslips were examined by SEM, which demonstrated smooth and pinhole free films, thereby confirming the AFM results. BgPS and TCPS surfaces were also examined with SEM and no topographical differences were observed. After 2 weeks in culture, the coatings remained intact, as observed by phase microscopy (not shown), although SEM sample preparation caused some cracks. We also observed no change in the surface topography of the 3D PLGA scaffolds, after 2 weeks in culture medium, as evidenced by SEM.

Bone marrow-derived cultures

Phase microscopy

PLGA-coated coverslips and TCPS and BgPS surfaces were seeded with bone marrow-derived cells and examined daily by phase microscopy for changes in morphology and bone nodule formation. Cells grown on both TCPS and PLGA surfaces showed a flattened morphology and were spread out. In comparison, fewer cells were observed attached to the BgPS substrate and these cells had mostly a rounded morphology. Bone nodules were initially observed on both the TCPS and PLGA surfaces at day 6. Bone nodules were absent at this time on BgPS.

Electron Microscopy

SEM was used to examine the bone-substrate interface after 2 weeks in culture. TCPS, a positive control, supported cement line deposition whereas BgPS, a negative control, did not (not shown). Cement line deposition, and collagen assembly, and mineralization were observed in this study on TCPS (not shown), as reported previously,[15] and PLGA surfaces (Fig. 2) but were absent from the BgPS surfaces (not shown). PLGA supported an elaborated cement line matrix with a thickness of approximately 0.5 μm [Fig. 2(a)]. An overlying cell layer and collagenous matrix were also observed [Fig. 2(b,e,f)]. Cultured PLGA 3D scaffolds were also observed by SEM (Fig. 3). Before cell seeding, the regular distribution of interconnected macropores that were separated from each other by struts of microporous polymer was evident [Fig. 3(a)]. After 2 weeks in culture, the polymer substrate was
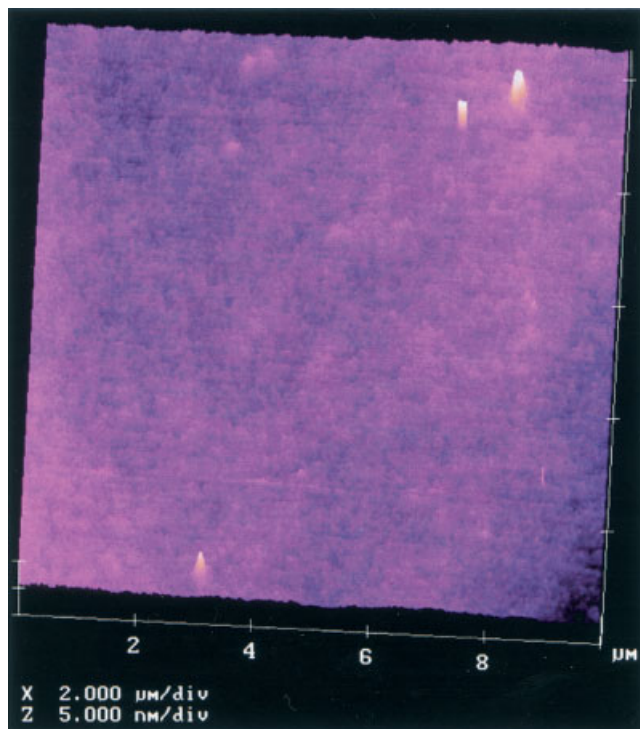

Figure 1. AFM of a PLGA 75:25 spin-coated glass coverslip demonstrate's that the substrate was essentially smooth. Field width = 10 microns. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].
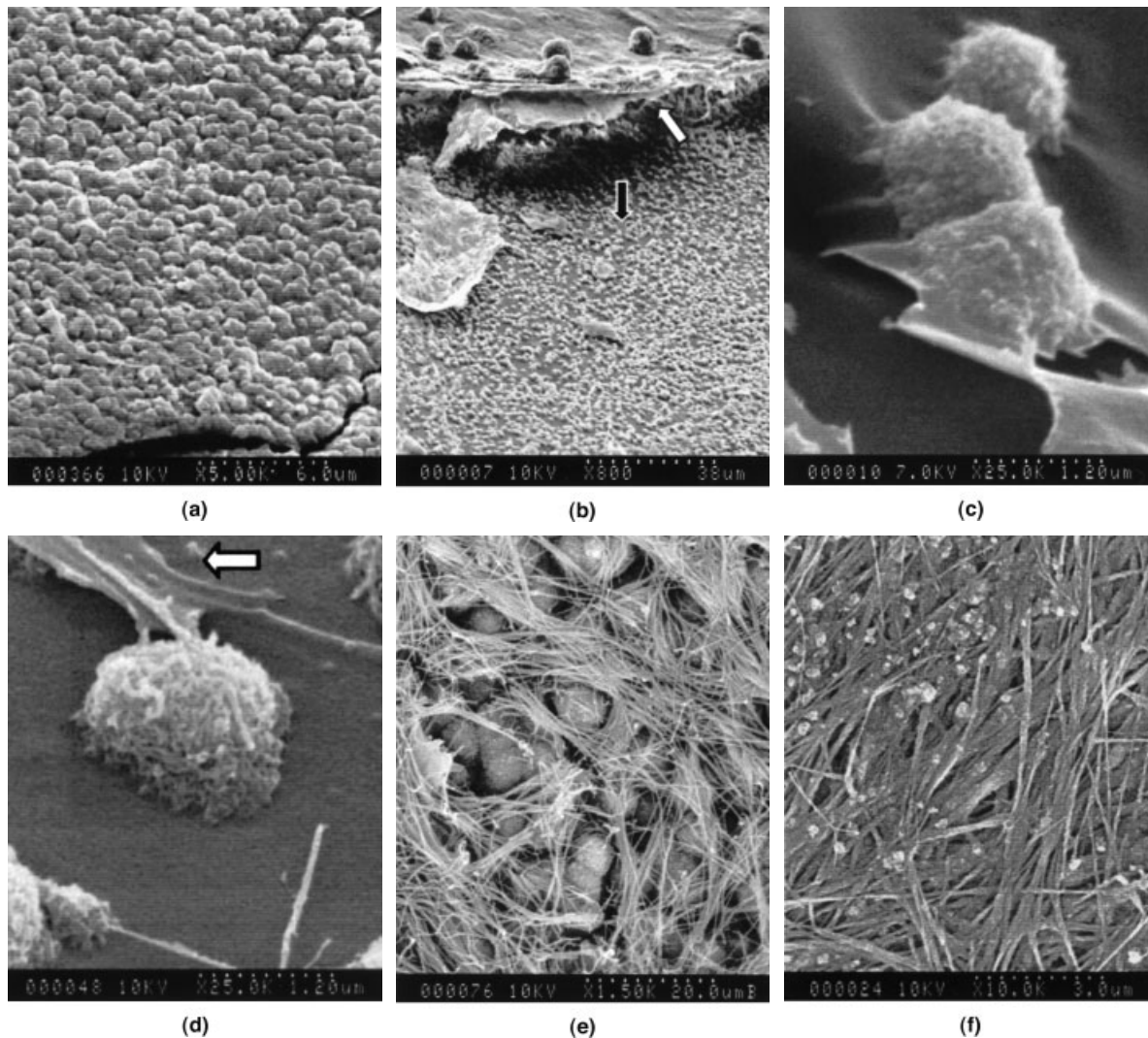
Figure 2. SEM display hallmarks of *in vitro* bone formation on a PLGA surface. (a) PLGA supported an elaborated cement line matrix. A small crack, most probably a result of SEM preparation, was observed, which gave us an indication of the thickness of the cement line matrix (approximately 0.5 μm). (b) Cement line globules were observed (black arrow) once most of the overlying cell and collagenous layer (white arrow) were removed with a blast of compressed air. (c) A tear in the PLGA coating, most probably as a result of SEM preparation, provided a close look at the attachment of the cement globules to the PLGA surface as well as the thickness of the PLGA spin-coated substrate (<100 nm). (d) After the cement globules were deposited on the substrate by cellular extensions (arrow), (e) collagen assembled and anchored directly to the underlying cement globules. (f) The collagen formed a confluent sheet and became mineralized, which was evident from the thickening of the collagen fibrils and globular accretions. These hallmarks of *in vitro* bone formation were also observed on the TCPS substrate.

covered with bone matrix and an overlying cell layer [Fig. 3(b)]. When the overlying cell layer and collagenous matrix were removed, an elaborated cement line matrix was revealed, which followed the tortuous interconnected PLGA scaffold geometry [Fig. 3(c)]. Examined by TEM (Fig. 4), these cement globules had similar morphology on both the TCPS and PLGA surfaces. Pseudopod extensions to, and collagen fibrils in the vicinity of, the globules were also evident.

EDS analysis determined that individual cement globules, in contact with the PLGA substrate, contained calcium (Ca) and phosphorous (P) [Fig. 5(a)]. These two elements were not observed in the non-

mineralized collagenous matrix [Fig. 5(b)]. The electron diffraction pattern obtained was characteristic of a poorly crystalline hydroxyapatite (not shown). The diffraction patterns of the cement globules and mineralized collagenous matrix were congruent.

DISCUSSION

For bone tissue-engineering applications, highly porous biodegradable scaffolds are being investigated as delivery vehicles for osteogenic cells.[19] During an ini-
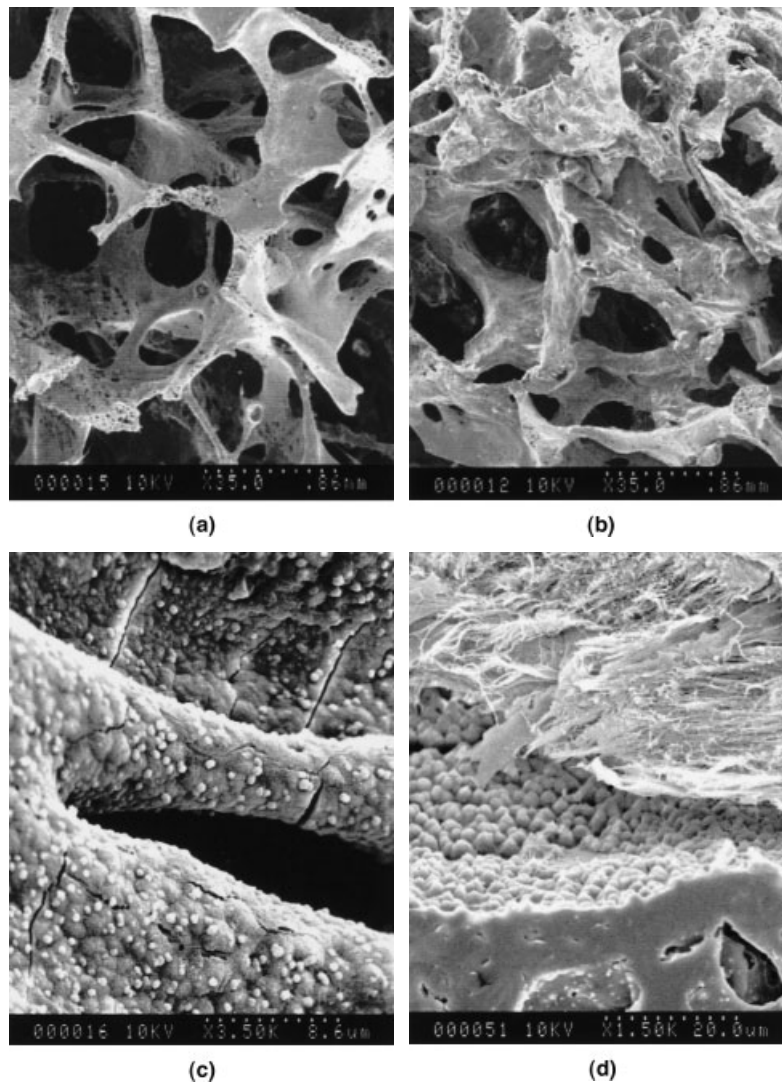
Figure 3. SEM of 3D PLGA scaffolds. (a) The native scaffold was highly porous with a great degree of interconnectivity. (b) After 2 weeks in culture, the cells had deposited matrix throughout the entire scaffold. (c) When the cell and collagenous matrix was removed, the cement line matrix was revealed adhering to the underlying PLGA substrate. In (d), we can observe the bulk PLGA scaffold in the foreground (white arrow), the cement line matrix, and the overlying collagenous matrix and cell layer (black arrow). Images reproduced, with permission, from Davies JE, Karp JM, Baksh D. Mesenchymal cell culture: Bone. In: Atala A, Lanza RP, editors. Methods of Tissue Engineering. San Diego: Academic Press; 2002. p-333–344.
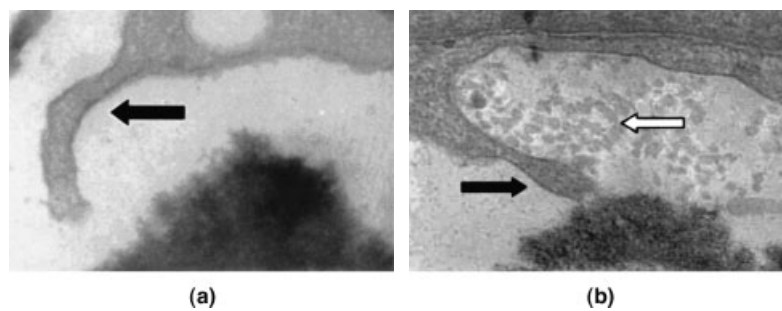

Figure 4. TEM of (a) TCPS (field width = 1.2 μm) and (b) PLGA (field width = 1.5 μm) surfaces. In both these micrographs, cement globules of similar morphology were observed. Also, pseudopod extensions were also evident in both micrographs (black arrows), most probably directly responsible for depositing the cement globules observed. Collagen fibrils were also evident (white arrows) through a cross-sectional view. Collagen fibrils, although evident on the TCPS substrate, were not evident in this micrograph (a), which most probably represents very early globule deposition compared to the globule in (b).
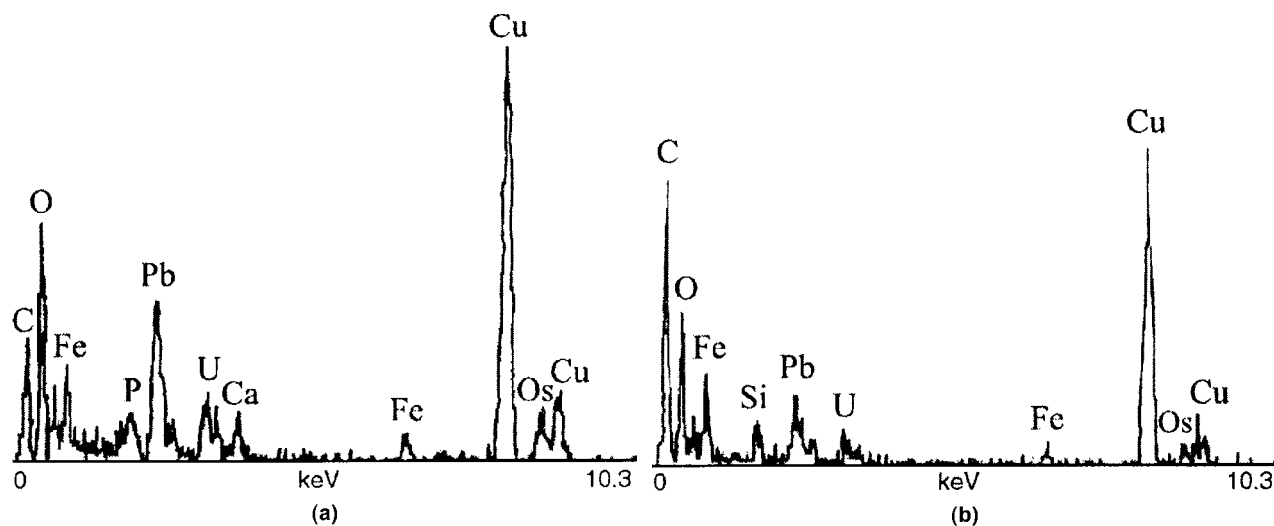
Figure 5. (a) Results from EDS reveal that the cement globules in contact with the PLGA surface contain calcium and phosphorus. (b) Analysis of the non-mineralized collagenous regions (osteoid) did not contain these minerals.

tial *in vitro* culture stage, cells colonize the scaffold surface and may begin to produce bone matrix. Our results indicate that rat marrow cells can ubiquitously produce bone matrix on the surface of our 3D scaffolds. Tissue-engineering scaffold surfaces, which support a cement line matrix, are advantageous because newly synthesized bone would rigidly attach to the substrate. We examined both 2D films and 3D scaffolds for the presence of interfacial matrix. 2D PLGA films cast into Teflon petri dishes facilitated examination of the interfacial matrix by TEM. These films were comparable, in terms of surface hydrophobicity by contact angle, elemental analysis by XPS and topography by SEM, as the spin-coated films (Table I). The creation of a smooth, pinhole-free PLGA surface was necessary to establish whether the cement line matrix could be elaborated on a pure PLGA surface in the absence of topographical features.

Because it has been demonstrated previously that BgPS does not support a confluent cement line matrix[20] whereas TCPS does,[21] these substrates were chosen as negative and positive controls, respectively. Our XPS and contact angle analyses indicated that the differences in cell attachment, spreading and matrix elaboration observed for BgPS and TCPS substrates, could be attributed to differences in surface energy rather than elemental composition (Table I). SEM observations indicated that there were no topographical differences between these two surfaces.

Because PLGA had a similar receding water contact angle (θ_R) to the BgPS negative control, and was substantially more hydrophobic relative to the TCPS positive control, we questioned whether PLGA could support the elaboration of a cement line matrix. We demonstrated that an afibrillar cement line matrix was directly apposed to the 2D PLGA films [Figs. 2(a–d) and 4(b)]. Furthermore, the same afibrillar cement line

matrix was directly apposed to the 3D PLGA scaffold surfaces [Fig. 3(c,d)]. Electron-dense globular accretions were observed in direct apposition to both PLGA (Figs. 2–4) and TCPS [Fig. 4(a)] surfaces, demonstrating a similar cellular response to PLGA samples and TCPS positive controls. Thus, despite the fact that PLGA is relatively hydrophobic, bone marrow-derived cells responded similarly to TCPS both morphologically and functionally. Because PLGA can support an elaborated cement line matrix, the overlying collagenous matrix and cell layer, by anchoring to the cement line, may be more firmly secured to the underlying PLGA substrate. It has been demonstrated that it is much more difficult to remove the cell layer and overlying collagenous matrix on surfaces which support an elaborated cement line matrix versus those that do not.[20]

The cement line is a mineralized matrix, consisting of calcium hydroxyapatite, which dissolves in acidic environments.[22] Because PLGA degrades to acidic products and calcium hydroxyapatite is susceptible to acidic degradation, we questioned the extent of mineralization at the interface. PLGA undergoes bulk (autocatalytic) degradation by simple hydrolysis into lactic acid and glycolic acid.[23] After the initial *in vitro* culture period described for tissue-engineering applications, which was the focus of our experiments, the degradation process would be accelerated upon transplantation in vivo by enzymes,[24] cellular activity, and cell-induced pH changes such as those caused by activated macrophages.[25] Because PLGA undergoes bulk degradation, the molecular weight of the polymer begins to decrease upon direct contact with water, but its mass does not change appreciably until the molecular chains are small enough to diffuse out of the polymer matrix,[26] thus the mass loss profiles are sigmoidal.[27] Böstman[28] and Agrawal et al.[29] have pos-

tulated that the delayed inflammatory response, which has been reported in many *in vivo* studies with PLA and PGA, may be related to the large release of acidic products. Lu et al. incubated thin and thick PLGA films in phosphate buffered saline and determined that increasing the thickness levels from 10 to 100 μm accelerated both the weight and molecular weight loss over a 10-week period.[30] At 2 weeks (the culture period for the present study) Lu et al. observed that the percent molecular weight remaining was approximately 85% for thin films and 65% for thick films of PLGA 75:25. At the same 2-week time point, Lu et al. also observed that the percent weight remaining was approximately 90% for thin films and 85% thick films of PLGA 75:25. Thus, it was assumed that films used in the present study degraded to some extent during the 2-week culture period. PLGA films used in the present study were between <100 nm for PLGA-coated coverslips and 400 μm for films cast in Teflon® petri dishes. All films, regardless of thickness, supported cement line matrix and remained intact during the culture period with no evidence of surface micropore formation. Our TEM analyses indicated that acidic polymer degradation products either did not affect the matrix or that such products had not formed during the 2-week culture period. Because the cement line matrix was not demineralized during the 2-week culture period, the collagen compartment above the cement line matrix would also remain mineralized, as evidenced by EDS analysis. In addition to the cement line matrix, collagen assembly and mineralization were also observed.

CONCLUSIONS

We conclude that both 2D PLGA films and 3D scaffolds can support an intact bone/polymer interface, in which mineralized cement line matrix is deposited directly on the polymer surface, and upon which the mineralized collagenous matrix of bone is elaborated. Thus, structural continuity between bone and this biodegradable polymer can be achieved.

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