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Maintenance of a bone collagen phenotype by osteoblast-like cells in 3D periodic porous titanium (Ti-6Al-4 V) structures fabricated by selective electron beam melting

Nikolas W. Hrabec¹, Peter Heinl², Rajendra K. Bordia¹, Carolin Körner², and Russell J. Fernandes³

¹Department of Materials Science and Engineering, University of Washington, Seattle, WA, USA

²Department of Materials Science, Institute of Science and Technology of Metals, University of Erlangen-Nuremberg, Erlangen, Germany

³Department of Orthopaedics and Sports Medicine, University of Washington, Seattle, WA, USA

Abstract

Regular 3D periodic porous Ti-6Al-4 V structures were fabricated by the selective electron beam melting method (EBM) over a range of relative densities (0.17–0.40) and pore sizes (500–1500 μm). Structures were seeded with human osteoblast-like cells (SAOS-2) and cultured for four weeks. Cells multiplied within these structures and extracellular matrix collagen content increased. Type I and type V collagens typically synthesized by osteoblasts were deposited in the newly formed matrix with time in culture. High magnification scanning electron microscopy revealed cells attached to surfaces on the interior of the structures with an increasingly fibrous matrix. The *in-vitro* results demonstrate that the novel EBM-processed porous structures, designed to address the effect of stress-shielding, are conducive to osteoblast attachment, proliferation and deposition of a collagenous matrix characteristic of bone.

Keywords

Extracellular matrix; orthopedic implants; SAOS-2; scaffold; type I collagen; type V collagen

Introduction

Stress shielding is a phenomenon observed in bone following replacement with load-bearing metal implants, for example, hip stems. A mismatch between the higher stiffness solid metallic implants and the more compliant bone leads to bone remodeling at the bone-implant interface (Wolff's Law) that can lead to bone loss and implant loosening (1). With the longer implant lifetimes becoming the norm, motivation to address stress shielding has been revived. Recent developments of lower-modulus hip stem implants, for example, the Zimmer Epoch hip stem that achieves a lower elastic modulus through a thin cobalt-chrome-molybdenum core and a thick polymeric outer layer, have reported clinical success up to 10 years post-implantation (2–4). The drive to design biocompatible implant materials with improved mechanical compatibility is justified.

Porous metals show promise in reducing the effects of stress shielding in orthopedic applications. The potential to optimize elastic modulus and yield strength through the control of structural properties such as relative density, pore size and strut size makes porous materials attractive to address this problem. Additionally, porous metals allow bone growth into the pores that could promote optimum osteo-integration and strengthen the bone-implant interface. Porous titanium is an obvious choice considering the proven biocompatibility of titanium alloys in orthopedic applications. Of the many previously developed methods for processing of porous titanium (5–7), selective electron beam melting (SEBM or EBM) provides a high degree of control over structural properties. This technique shows great promise in being able to realize structures with the complex gradients of porosity that recent modeling work has recommended is necessary to optimally address stress shielding (8). The EBM process is a solid freeform fabrication method (SFF) capable of building three-dimensional structures layer by layer. Loose powder is applied one layer at a time to a start plate. After spreading each layer of loose powder, an electron beam sinters or melts select areas according to computer-aided design (CAD) files. The CAD model, as well as tunable build parameters (electron beam current, electron beam scanning speed, energy input per unit length and layer thickness), can affect the structure morphology. Most current EBM porous titanium studies (9–13), have focused on the processing, structural and mechanical characterization. The only *in-vitro* characterization study on EBM porous titanium, by Ponader, et al. (14), showed that human fetal osteoblast-like cells proliferated very slowly in porous titanium scaffolds compared to non-porous compact discs of the same material raising questions if the pores in the titanium structures created a microenvironment unsuitable for cellular attachment. An analysis of bone specific gene expression or protein synthesis by the cells proliferating within the porous titanium constructs was not able to be evaluated in this study (14).

We have focused on the biochemical characterization and *in-vitro* behavior of bone cells (osteoblasts) cultured in these EBM Ti-6Al-4 V scaffolds as a step toward long-term clinical implant studies. Osteoblasts synthesize a collagenous matrix and regulate mineralization of the matrix by releasing membrane-bound matrix vesicles concentrated with calcium and phosphate (15). Collagen is the most prevalent protein in bone, comprising 85–90% of protein and is responsible for bone structure and strength. Type I collagen is the major collagen in bone matrix and forms cross-linked hetero-fibrils with type V collagen, a minor but important component. There is increasing evidence that type V collagen forms a template upon which type I collagen molecules are deposited in bone to form the large diameter collagen fibrils observed by electron microscopy (16,17). Besides functioning as the main structural component of vertebrate bone, fibrils serve as sites for biomineralization – the deposition of hydroxyapatite in the organic bone extracellular matrix (18). Utilizing a well-characterized human osteoblast-like cell line (SAOS-2) (19,20) the collagen deposited within the extracellular matrix elaborated by these cells when seeded within the Ti-6Al-4 V scaffolds was biochemically and ultrastructurally analyzed.

Materials and methods

Scaffold processing

Inert gas atomized, pre-alloyed Ti-6Al-4 V powder (spherical, average particle size 70 μm) was used with an EBM S12 system (Arcam AB, Mölndal, Sweden) to fabricate structures according to a previously developed processing method (11,12). A CAD model that mimics the crystal structure of diamond was used (21), and four structures were made over a range of relative densities, pore sizes and strut sizes using the build parameters in Table 1. Pore size was controlled by changing the scale of the CAD model. Strut thickness was controlled by changing the energy input. The energy input was changed by varying beam current, and

all other build parameters were held constant for all structures. We have published further details on the processing of these scaffolds (22).

Structural characterization

Relative density was measured via the Archimedes method (ME3360 Archimedes system and AE100 mass balance, Mettler Toledo, Switzerland). X-ray micro-computed tomography (microCT) was used to obtain average (and median) pore and strut size as well as specific surface area. Structures were scanned at a resolution of 10 μm (MicroCT 40, Scanco Medical, Switzerland, automated thresholding). Specific surface area was calculated from microCT results using the total volume of the structure, including porosity. It should be noted that the values for surface area are suitable for use in comparing to the other structures of this work but not as absolute values due to the resolution of the scan. Pore and strut morphology was observed using SEM (JSM7000F, JEOL, Tokyo, Japan).

Scaffold preparation

Cylindrical scaffolds approximately 15 mm in diameter and 4 mm in height were cut into quarters to fit in a 48-well plate. The 4 mm height ensured at least two pores in the height dimension for all structures. All scaffolds were passivated according to ASTM F86: Surface Preparation and Marking of Metallic Surgical Implants. In this procedure, the samples are immersed in 30% HNO_3 for 30 min, followed by thorough washing in tap water and sonication in a 50/50 mixture of ethanol and de-ionized (DI) water. The mass of each scaffold was recorded, and microCT data was used to calculate the volume and surface area of each scaffold. Sterilization of the scaffolds was performed in a biological safety hood by soaking in ethanol (100%) until the ethanol had completely evaporated followed by UV exposure for three hours.

Cell culture

Human osteosarcoma cells (SAOS-2 cell line, ATTC # HTB 85) were seeded on the scaffolds (10^5 cells/scaffold) and incubated at 37 °C and 5% CO_2 in air. After three hours, 0.5 mL of complete medium was added to each well. Complete medium consisted of McCoy's 5 A media (Gibco Invitrogen, Grand Island, NY) with 10% fetal bovine serum (FBS, Gibco Invitrogen, Grand Island, NY) and 10 $\mu\text{g/mL}$ ascorbate. Cells were cultured at 37 °C and 5% CO_2 in air for one to four weeks. Proliferation assays, biochemical assays, and SEM characterization were performed at one week intervals up to four weeks. Medium was changed every 2–3 d for the duration of the experiment.

Cell proliferation

To determine if cells seeded in scaffolds multiplied in culture, cell proliferation was assayed by MTS (Promega CellTiter96 AQueous, Madison, WI) and hemocytometry. Cell seeded scaffolds were assayed in triplicate at each time point for each of the four EBM structures. After carefully removing the medium, each scaffold was transferred to a new well ensuring only cells attached to the structure would be counted. Forty microliters MTS and 0.5 mL fresh, complete medium was added to each well, and the scaffolds were incubated for four hours. After incubation, three aliquots of 100 μL were taken from each well and added to separate wells in a 96-well plate and the absorbance read at 490 nm using a plate reader. The absorbance units were used to compare relative number of cells between different structures and at different time points. The absorbance of a given scaffold was reported as the average of the three aliquots taken from that scaffold. The absorbance for a given structure for one time point is reported as the average of three scaffolds. MTS measurements for each scaffold were normalized by the scaffold volume to account for slight differences in scaffold size.

Biochemical analysis

Scaffold preparation—Three scaffolds were analyzed for each EBM structure, at each time point. After media was removed from the wells and from within the pores of the scaffolds, they were frozen at -20°C until analyzed.

Extraction of collagen—Newly synthesized and cross-linked collagen deposited in the extracellular matrix of the SAOS-2 cells was extracted by digesting with 0.1 mg/mL pepsin in 0.5 M acetic acid (0.5 mL) for 24 h at 4°C (19).

Estimation of collagen content—The content of collagen in the scaffolds was estimated by measuring the content of hydroxyproline. Aliquots of pepsin extract were hydrolyzed in 6 M HCl, 100°C for 24 h. The hydrolysate was colorimetrically assayed for hydroxyproline and collagen content established as described previously (19,23). Collagen content was expressed as milligrams per scaffold, and to be consistent with the proliferation assay, amount of collagen for each scaffold was normalized by the volume of that scaffold.

Identification of collagen types—To determine if the attached cells could synthesize and deposit collagen typical of bone, collagen deposited in the extracellular matrix of the cell layer (after weeks 1, 2 and 3 in culture) was solubilized with pepsin and identified by 6% polyacrylamide gel electrophoresis (SDS-PAGE) (19,24). Equal aliquots were dried, dissolved in Laemmli sample buffer with dithiothreitol (a disulfide bond reducing agent) and heated at 100°C for three minutes. Solubilized collagen chains were visualized by Coomassie blue staining. Purified human bone type I collagen and purified SAOS-2 type V collagen were used as standards. A pepsin extract of collagen from SAOS-2 cells cultured in monolayer on tissue-culture plastic was also used as a control (19). Collagen chains in samples were identified by comparing their electrophoretic migration to the migration of the collagen chains in the standards.

Investigation of cell and extracellular matrix morphology by SEM

To visualize attachment of cells on as-built Ti-6Al-4 V, a cell seeded scaffold of each porous structure at all time points was observed via SEM with energy dispersive spectroscopy (EDS). The cells in the scaffolds were fixed overnight using Karnovsky's fixative (2% Glutaraldehyde, 2% Paraformaldehyde), dehydrated using increasing concentrations of ethanol in DI water (up to 100% ethanol), further dried using critical point drying, and sputter coated with platinum (~5 nm thickness). Electron Dispersive Spectroscopy (EDS) was used to detect organic carbon from cells and extracellular matrix.

Results

Structural characterization

From microCT results, average pore and strut sizes as well as specific surface area are shown in Table 2 for the four structures investigated. Median pore and strut sizes are also shown as it is felt these give a more accurate representation of the characteristic feature size. From these results it is clear that, as expected, scaling of the CAD model resulted in a change in pore size but had relatively little effect on strut size. These results also show the expected effect of a higher energy input as structure 4 had a relatively larger strut thickness, compared to the other three structures, which all had a lower energy input. SEM observation of these structures (Figure 1) showed sintered particles (spherical, average size $70\text{ }\mu\text{m}$) and texture lines due to melt movement during layered building were abundant on the strut surfaces of all structures. Further details and results on the distribution of pore and strut sizes for these structures are presented elsewhere (21). The mechanical properties of these

structures were found to be in the range for cortical and cancellous bone. Elastic modulus values for the four structures were as follows: S1 6.40 ± 0.07 , S2 0.89 ± 0.01 , S3 3.59 ± 0.01 and S4 6.79 ± 0.06 (all in GPa) (21). Elastic modulus of normal cancellous bone is 0.2–2.0 GPa and cortical bone ~ 15.2 GPa (25).

Cell proliferation

Results of the MTS proliferation assay, normalized by the volume of each structure, are shown in Figure 2. The general trend observed for all structures is an increase in the number of cells between week 1 and 2, followed by a plateau in cell proliferation from week 2 to 4. The construct with intermediate surface area, pore size and strut density with an elastic modulus closer to the upper range of normal cancellous bone (S3) showed greatest proliferation implying favorable conditions compared to the other porous constructs. If the standard deviations are considered, no significant differences in attachment or proliferation rate were observed when comparing the four structures analyzed. These general trends were also seen in the hemocytometry data (not shown).

Collagen content

Volume-normalized results for collagen content are shown in Figure 3. Total collagen content appears to increase with time in culture for all structures. No differences between the total collagen content in the various structures can be discerned. The average amount of collagen, before normalizing by scaffold volume, was approximately 0.07 mg/scaffold. The construct that had the smallest pore and strut size and highest surface area (S1) showed maximum collagen accumulation after four weeks in culture. The construct that had the highest cellular proliferation (S3) showed comparable collagen content to S1 by 3 weeks in culture.

Identification of collagen types

To identify the types of collagen deposited in the extracellular matrix, pepsin solubilized collagen was examined by gel electrophoresis. Component protein chains of type I and type V collagen were identified based on electrophoretic migration as shown in Figure 4, when compared to standards of purified type I and type V collagen. Nearly equal proportions of type I and type V collagens were found deposited in the extracellular matrix of all structures after three weeks. A pepsin extract of collagen from SAOS-2 cells cultured in monolayer (19) was used as a standard (“SAOS-2” lane in Figure 4) and also showed comparable ratios of type I (48%) and type V (52%) collagen. Since equivalent aliquots of collagenous extracts were loaded per experimental lane for gel electrophoresis, band intensities were used as a qualitative indication of relative amount of pepsin-resistant collagen chains and used to compare from one lane to another. For all structures, it is observed that pepsin resistant type I and V collagen increased consistently with time in culture. Structure 4 had the most pepsin resistant type I and V collagen for week 2 and 3 as can be seen by the relatively intense collagen bands (Figure 4). Structure 3 had the most pepsin resistant collagen at week 3.

Scanning electron microscopy

SEM enabled the observation of the cellular and extracellular matrix morphology within the porous Ti-6Al-4 V structures. Based on increased cell proliferation and high collagen content only the S3 structure is documented here (Figure 5) although cells were clearly seen attached to all the porous structures (data not shown). A fibrous extracellular matrix was observed with increasing time in culture. Comparing the surfaces of unseeded S3 structure (Figure 5a) to cell seeded S3 structure (Figure 5b), a clear distinction between bare titanium and the cell layer was observed after three weeks in culture. Energy dispersive spectroscopy (EDS) verifies this distinction (Figure 5 insets) as cellular structures have considerably more

carbon than the bare Ti-6Al-4 V substrate. EDS was unable to detect calcium or phosphorus indicating the absence of mineralization in the cultures. Cells with both round and flat morphologies were observed (Figure 6A). Size was used to distinguish between rounded cells (~10 μm for SAOS-2) and sintered particles of titanium (~75 μm). The surface texture of rounded cells also helped distinguish them from the smoother surfaces of sintered titanium particles. With time in culture, the surfaces of the Ti-6Al-4 V structures were covered with cellular material and distinguishing cells from extracellular matrix was difficult. It was very obvious that cells could form multiple layers and span pores up to 30 μm as is clearly seen in Figure 6(A).

Some cells were observed to have protrusions from the cell membrane (Figure 6B). These features are reminiscent of vesicles being secreted by the cells and are signs of dynamic biosynthetic and secretory activity (26). Figure 6(B) also clearly shows cell filopodia, which are often observed in cell-cultured osteoblasts (27–30).

By SEM, no difference was observed in the number of attached cells for a given time point when comparing all four structures, and this was reflected in the cell proliferation data. Also, there was no discernable difference in the number of cells as a function of time for any of the structures. However, the multilayering of cells did make such observations difficult. Figure 7 shows the surface density of cells for S3 structure at each time point. The SEM images clearly show that by a week in culture, cells are attached to the surface and display a flattened, polygonal morphology (Figure 7a). With increased time in culture, from week 2 to week 4, the cell layer begins to accumulate extracellular matrix revealing a more amorphous and fibrillar appearance (Figure 7b–d). By week 4, cells are difficult to distinguish from the extracellular matrix that now clearly shows the presence of fibrillar elements (Figure 7d). Such fibrillar elements have been observed previously in osteoblast cultures (29). It is important to note here that type I and V collagen content also increases from week 1 to week 3 (Figure 4). These results for structure 3 are representative of the other three structures.

Figure 8 examines the observed fibrils at week 4 within structure 3 at higher magnification. Here, the fibrous nature of the extracellular matrix is more apparent. In Figure 8(a), one can appreciate the considerable length of these collagen fibrils (greater than 20 μm in some cases) in the extracellular matrix. The cell outline with pseudopodia is clearly discernable from collagen micro-fibrils, as shown in Figure 8(c). As seen in Figure 8(d), by week 4 in culture, collagen micro-fibrils were in some cases arranged into fibrils. A fibrillar meshwork was also seen occasionally in the extracellular matrix (Figure 9b), surrounding cells of rounded morphology (Figure 9a). Whether this is a meshwork of collagen fibrils has not been determined, but similar structures have been observed previously for osteoblasts in culture (31). Further, this meshwork is different from that seen on flattened cells (Figure 8d). No difference could be observed in relative amount of fibrils at a given time point when comparing the four structures to each other.

Discussion

The choice to use human SAOS-2 osteoblast-like cell line rather than mammalian primary osteoblasts in this study was made for two reasons: (i) the cell line has a stable phenotype and is easy to culture and (ii) this cell line has been well documented in the field of bone biology and shown to have a mature osteoblast phenotype. Rodan et al. (32) in characterizing the cell line concluded that the SAOS-2 cells shared several features of primary osteoblasts including high alkaline phosphatase activity, typical binding of 1–25 dihydroxyvitaminD3 to the 3.2 S receptor protein, sensitive adenylate cyclase response to parathyroid hormone, osteonectin synthesis and calcification of the extracellular matrix typical of woven bone. Since then, the SAOS-2 cells have been shown to synthesize

integrins that support attachment to implant materials including solid Ti-6Al-4 V (33) and have cytokine and growth factor expression similar to normal primary osteoblasts (34). Recent studies have demonstrated that SAOS-2 cells can be induced to express osteoprotegerin following treatment with sphingosine-1-phosphate (35) and also express the estrogen receptors α and β (36). We have analyzed the collagen synthesized by the cell line in detail and have shown that four-fifths of the collagen synthesized was type I collagen and one-fifth type V. Type I collagen deposited in the extracellular matrix is cross-linked by hydroxylysyl pyridinoline, the major cross-link found in bone (19).

Measurement of bone specific gene expression on EBM-processed Ti-6Al-4 V scaffolds have been unsuccessful due to insufficient number of attached cells (14). So not surprisingly, direct measurement of total collagen protein content and the maintenance of bone collagen phenotype by osteoblasts in EBM-processed Ti-6Al-4 V scaffolds had not yet been addressed. Whether the EBM technique or the difference in porosity of the scaffolds contributed to the inadequate attachment of cells was questioned. We show here that SAOS-2 osteoblast-like cells can attach and proliferate within EBM processed porous scaffolds of all pore sizes tested (Figure 2). Further, total collagen content increased with time in culture for all porous structures (Figure 3). This suggests that (i) the EBM technique did not change the surface properties of the structures and (ii) the SAOS-2 cells that attached to the titanium structures are metabolically active and synthesize and deposit collagen molecules in the extracellular matrix as typically expected for bone forming cells. The average amount of collagen per scaffold (0.07 mg) per total surface area for any of these scaffolds (largest possible is 6 cm²) is approximately 0.01 mg/cm². This is approximately twice the previously reported collagen production for SAOS-2 cells cultured in monolayer on polystyrene culture dishes (0.0052 mg/cm²) (19).

It is apparent from Figure 4, that type I and V collagen are the principal components of the extracellular matrix as in human bone (37) and there is more present in later weeks as compared to earlier weeks. This is generally corroborated by increased total collagen content and by SEM of the extracellular matrix deposited in the structures. The proportion of type I to type V collagen was comparable to that found in the pepsin extracted collagenous matrix of SAOS-2 cells after one month monolayer culture (48% type I and 52% type V). It must be noted here that in monolayer culture, type I collagen is still the major collagen synthesized by the SAOS-2 cells accounting for 80% of the medium and cell layer collagen and type V accounting for 20%. Most of the type I collagen is found in the medium, and roughly equal amounts of type I and type V collagen were recovered in the pepsin extract of the cell layer (19). High proportions of type V to type I collagen have been shown to accumulate in the cell layers of primary chicken osteoblasts after a month in culture (38). Since type V collagen forms a template upon which type I collagen molecules are deposited in bone to form the large diameter collagen fibrils observed by electron microscopy (16,17) having most of the type V collagen deposited in the bone matrix during early stages of culture by osteoblasts may not be unusual for correct type I collagen fibrillogenesis. It is reasonable to speculate here that with time in culture, type I collagen will be deposited on the type V-type I heterofibrils in increasing amounts to form larger diameter fibrils as has been previously been reported for primary chicken osteoblast cultures (38). Clearly in Figure 7, we see the presence of higher ordered fibrillar elements in S3 structures indicating that collagen fibrils have assembled. By week 4 in culture thicker diameter fibrils are very apparent. Similar fibrillar collagenous structures have been previously observed in osteoblast cultures by electron microscopy (29,31,38).

Besides forming the main structural architecture in bone, collagen fibrils serve as sites for the deposition of hydroxyapatite in the organic matrix. Although collagen fibrils are deposited in the extracellular matrix in the Ti-6Al-4 V scaffolds, mineralization was not

observed by SEM. The EDS spectra showing a lack of calcium or phosphorus (Figure 5) in the matrix supports this observation. This was an expected result since optimal conditions for mineralization of SAOS-2 cell cultures (addition of beta glycerol phosphate (β -GP)) (39–41) was not used. Experiments were specifically designed to evaluate cell proliferation and collagen synthesis since strength of calcified tissue depends in part on the molecular composition, structure and native organization of the collagenous matrix synthesized and laid down by the osteoblasts (18). The SEM data also support the proliferation and biochemical observations since cells and collagen fibrils were seen both in the interior of the scaffold structures as well as on the exterior. The presence of cellular features such as filopodia and protrusions from the cell membrane (Figure 7) suggests healthy cell secretory activity typical for osteoblast cultures (26–30).

It has been hypothesized that bone remodeling due to stress shielding contributes either directly or indirectly to implant loosening (42–46). Recent clinical studies (47,48) have not shown any evidence to support this hypothesis and imply that the major cause of aseptic implant loosening is wear debris created by articulating implant surfaces (46,49). However, Huiskes has maintained that with longer implant lifetimes, bone remodeling due to stress shielding will become an important issue making revision surgeries more difficult (46). The EBM-processed scaffolds described here have mechanical properties within the range of cortical and cancellous bone (21) and is in concurrence with recent observations by Li et al. (50). Porous titanium scaffolds could be effective for use in physiologically optimized load bearing and non-load bearing bone replacement implants. The mechanical properties coupled with the encouraging biochemical and ultrastructural results promises improved osteo-integration and minimal stress-shielding and could reduce the need for revision surgery.

Conclusions

Three-dimensional periodic porous Ti-6Al-4 V structures emulating the diamond unit cell were fabricated using EBM over a range of relative densities (0.17–0.40) and pore sizes (~500–1500 μ m) by varying the scaling of the CAD model and the energy input. Human osteoblast-like cells were found to attach and proliferate on these structures as determined by proliferation assays and this was supported by SEM observation of cells attached on interior surfaces of the porous structures. Attached cells were shown to maintain a bone collagen phenotype synthesizing type I and type V collagen. Ultrastructurally, over time in culture a fibrillar collagenous network was observed by SEM. The EBM-processed Ti-6Al-4 V structures show significant promise as porous biocompatible implants that can be optimally designed for load-bearing bone replacements. This *in-vitro* study demonstrates that a collagenous extracellular matrix can be deposited not only on the surface but more importantly within the pores of these structures. The results support implant design using EBM-processed Ti-6Al-4 V structures for *in-vivo* studies in large animal models.

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Declaration of interest

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Figure 1. SEM images of (a) structure 2 and (b) structure 4 showing sintered particles (white arrows) and texture lines (black arrows) from layered building on the surfaces of struts.

Figure 2.

In-vitro proliferation (MTS) results are shown normalized by scaffold volume. Average standard deviations were as follows: (S1) 1.1, (S2) 0.8, (S3) 1.5 and (S4) 0.6.

Figure 3. Total collagen content results are shown for the hydroxyproline assay, normalized by scaffold volume. Average standard deviations were as follows: (S1) 0.08, (S2) 0.05, (S3) 0.12 and (S4) 0.09.

Figure 4.

Gel electrophoresis results are shown using the following notation: “s” =structure, “w” =week, “typeI” is a purified human bone type I collagen standard, “typeV” is a purified SAOS-2 type V collagen standard, “SAOS-2” is a pepsin extract of collagen from SAOS-2 cells cultured in monolayer used as a standard, and “MW” is a globular proteins molecular weight standard. For all structures, type I and V collagen are present in increasing amounts up to week 3.

Figure 5.

SEM images comparing (a) bare Ti-6Al-4 V structure 3 surface without cells showing expected Ti-6Al-4 V EDS spectra (inset) with lower carbon content than (b) Ti-6Al-4 V structure 3 surface seeded with cells after three weeks in culture showing Ti-6Al-4 V EDS spectra (inset) with higher carbon content from cells attached to the Ti-6Al-4 V surface. Black boxes in (a) and (b) denote areas where EDS spectra was sampled. Presence of Pt is from sputtered conductive coating prior to SEM observation.

Figure 6.
(A) SEM image showing examples of round (white arrow) and flat (black arrow) cell morphologies. Both morphologies are approximately 10 μm in diameter, as expected. (B) SEM images of (a) lower and (b) higher magnification of the same cell showing cell secretion remnants (black arrow) and filopodia (white arrow).

Figure 7.

SEM images taken for structure 3 scaffolds after (a) one week, (b) two weeks, (c) three weeks, and (d) four weeks in culture. There is no distinguishable difference in surface density of cells. The white arrow in (d) week 4 is pointing to fibrillar elements.

Figure 8.

SEM Images of week 3 on structure 3 of (a) low magnification of a flat cell with collagen fibrils, (b) higher magnification of area bounded by rectangle in “a”, (c) higher magnification image of area bounded by rectangle in “b” showing entangled, individual collagen micro-fibrils (white arrow) compared to cell pseudopodia (black arrow), (d) higher magnification image of area bounded by rectangle in “b” showing a collagen fibril composed of several micro-fibrils (white arrow).

Figure 9.

SEM images showing a fibrous meshwork (white arrows) at week 3 on structure 3. (a) SEM image showing the fibrous meshwork surrounding a cell of rounded morphology, (b) higher magnification SEM image of the same fibrous meshwork providing more detail of the fibrous nature of this feature. Whether this is a collagenous meshwork is not clear.

Table 1

Processing parameters for Ti-6Al-4 V EBM structures.

Structure	Scaling of CAD model	Beam current (mA)	Beam speed (mm/s)	Acceleration voltage (kV)	Energy input per unit length (J/mm)	Layer thickness (µm)
1	0.6013	2	240	60	0.5	70
2	1.0915	2	240	60	0.5	70
3	0.7239	2	240	60	0.5	70
4	0.75	2	125	60	0.96	70

Table 2

Structural parameters for EBM structures.

Structure	Scaling of CAD model	Energy input per unit length (J/mm)	Relative density	Average (median) pore size (μm)	Std. dev. (μm)	Average (median) strut size (μm)	Std. dev. (μm)	“Specific surface area” surface area/total volume (/cm)
1	0.6013	0.5	0.39	525 (570)	142	398 (430)	111	33.68
2	1.0915	0.5	0.17	1428 (1540)	327	444 (460)	117	14.49
3	0.7239	0.5	0.30	724 (790)	190	412 (440)	108	27.23
4	0.75	0.96	0.40	633 (680)	174	530 (570)	148	28.53