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BONE SCAFFOLD FABRICATION USING POROGEN BASED INJECTION MOLDING METHOD AND BIOCOMPOSITE MATERIALS

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Abstract: Drop on demand printing (DDP) is a solid freeform fabrication (SFF) technique capable of generating physical features required for scaffolds to be used in hard tissue repair. Here we report results toward the development of a reproducible manufacturing process for tissue engineering scaffolds based on injectable porogens fabricated by DDP. Thermoplastic porogens were designed using Pro/Engineer and fabricated with a commercially available DDP machine. Scaffolds composed of either pure polycaprolactone (PCL) or homogeneous composites of PCL and calcium phosphate (CaP, 10% or 20% w/w) were subsequently fabricated by injection molding of molten polymer-ceramic composites. The precisely formed scaffolds were separated from the porogens in an agitated ethanol bath. Attainable scaffold pore sizes using the porogen-based method were found to be 200 μm for pure PCL. We characterized the compressive strength of 90:10 and 80:20 PCL-CaP composite materials (19.5+/-1.4 MPa and 24.8+/-1.3MPa respectively) according to ASTM standards, as wells as pure PCL scaffolds (13+/-1.2 MPa) fabricated using our process. Initial cell-biomaterial interaction studies demonstrated that our PCL and 80:20 PCL-CaP composite scaffolds supported attachment and proliferation of human embryonic palatal mesenchymal (HEPM) cells, as evidenced by fluorescent nuclear staining and the Alamar Blue™ assay. Scanning electron microscopy (SEM) revealed that HEPM cells spread and demonstrated histiotypic mesenchymal morphology.

Keywords: *Drop on demand printing (DDP), Porogen-based, Polycaprolactone (PCL), Calcium Phosphate (CaP)*

1. Introduction

Tissue engineering is an interdisciplinary field that draws from materials science, cell biology, and biotechnology to synthesize effective strategies for repair or replacement of damaged or diseased tissues[1]. Typically, *in vitro* bone tissue engineering uses engineered 3-D scaffolds[2], made of synthetic biodegradable polymers[3] or bioceramics[4], as substrates for 3-D culture of osteoblasts or other applicable cell types.

The recent application of solid freeform fabrication (SFF) to manufacturing scaffolds for tissue engineering[5-8] is limited by the fact that SFF machines must be adapted to the fluid mechanical properties of each biomaterial under consideration. For drop-on-demand- printing(DDP) and fused deposition modeling, the machine parameters must match the physical properties of the build material, i.e. viscosity and surface tension. These properties vary greatly amongst different biomaterials, making the use of a single machine for direct fabrication of scaffolds from multiple biomaterials difficult. Therefore it is desirable to develop SFF fabrication processes in which a single, universal porogen material is used to build porogens that may then be injected with a wide range of biomaterials.

The innate rigidity of the synthetic biodegradable polymer polycaprolactone (PCL) makes this material well suited for the fabrication of tissue engineering scaffolds, mainly for orthopedic applications[9,10]. Calcium phosphate(CaP), a major constituent of native extracellular matrix(ECM) in bone[11], is frequently used as a scaffold material for bone tissue engineering[12-14]. In the past, we designed and implemented a thermoplastic porogen-based process for the fabrication of cytocompatible injection molded calcium phosphate cement (CPC) scaffolds[15]. In extending these studies, we now used two well-defined biomaterials, PCL and CaP, to generate cytocompatible scaffolds with precise architectural features and appropriate mechanical properties for hard tissue repair by injection molding of thermoplastic porogens fabricated by DDP.

2. Materials and Methods

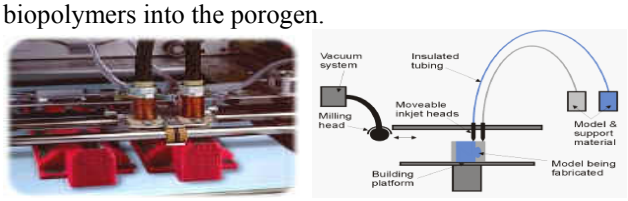
2.1. Drop-on-Demand Printing Machine

All thermoplastic porogens were fabricated using a commercial DDP machine (Solidscape™ ModelMaker II,) based on thermoplastic ink jetting technology (Figure 1). Porogens were built based on a simplified CAD model generated using Pro/Engineer™ (Figure 2).

2.2 Structured Porogen Design

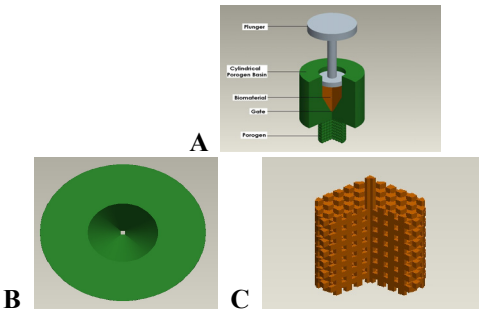
The Pro/Engineer™-designed injectable porogen model with 100% interconnectivity is shown in Figure 3. Each void of the square scaffold is in the shape of a cube and is separated from adjacent voids by struts on four of its sides. Scaffolds were designed with pore sizes of 600μm, 400μm, 300μm and 200μm. In previous studies, generation of biocompatible scaffolds using injectable porogens has been accomplished by polymer solution casting[6]. However, most of the solvents which are commonly used to solubilize synthetic biopolymers, such as dimethyl formamide, chloroform, and dioxane, will also dissolve the proprietary thermoplastic material used with the Solidscape™ machine, making solution casting

difficult to implement in our process. Therefore, in order to use the parts fabricated by the machine without any secondary processing[6], we chose to inject molten biopolymers into the porogen.



A B

Figure 1: Diagram of Solidscape Modelmaker II RP system A: Inkjet printhead; B: Process diagram.



A

B C

Figure 2: Computer generated models of the scaffold porogen. A: Injectable porogen; B: Overhead view shows the single injection gate; C: Resultant scaffold consisting of pores (200, 300, 400, or 600 m^3).

In order to empirically determine the minimum porogen basin wall thickness and maximum biomaterial injection temperature for which thermoplastic porogens would consistently maintain structural integrity, simple destructive testing was conducted. Based on these preliminary experiments a 3.18mm wall thickness and a biomaterial injection temperature of 75°C were selected. In order to minimize air entrapment and weld line formation, the porogen was designed such that molten biomaterial would flow into the cavities of the porogen through a single injection gate (Figure 2B). The dimensions of the port were equal to the pore size of the particular scaffold being injected. Therefore a transition region was needed to go from a relatively large basin where molten material could be deposited down to the gate dimension corresponding to the desired pore size of the scaffold (Figure 2B). The interior diameter of the basin was designed such that the plunger of a standard plastic 1 ml syringe could be used to force the molten biomaterial into the cavities of the porogen (Figure 2A).

2.3 Fabrication of Scaffolds:

Following fabrication of structured thermoplastic porogens, scaffolds were generated by injection molding as described below. The overall process is illustrated in Figure 2A.

PCL Scaffolds: PCL pellets (average molecular weight = 65,000, Sigma) were melted in an oven (VWR 1410) at 75ºC. Concomitantly, the porogens were also preheated to 75C. Molten PCL was drawn into a 1 ml syringe. The flat tip of the syringe was placed into the basin, thus allowing the plunger to advance from the syringe body into the basin of the porogen (Figure 2A). The syringe was emptied quickly and the filled porogen was allowed to cool. After solidifying, excess PCL was trimmed. In order to separate the porogen from the scaffold structure after biomaterial solidification, the filled porogens were immersed into 99% ethanol (Fisher) in a 10ml test tube. The tube was shaken vigorously and the solvent replaced every 15-20 seconds, until all porogen material was dissolved, as evaluated by the colorless appearance of the solvent. After porogen removal, the scaffolds were then allowed to air-dry at room temperature. A cutout view of the scaffold structure corresponding to the porogen design is shown in Figure 2C.

PCL-CaP Composite Scaffolds: PCL-CaP composite scaffolds were fabricated in the same fashion as the PCL scaffolds, with the additional step of preparing the PCL-CaP composite. For that, dry PCL pellets and CaP powders were weighed and mixed at the desired ratios in an aluminum specimen dish. After melting at 75ºC, the mixture was homogenized using an ultrasonic probe, and reheated as necessary; total mixing time was about 30 minutes. Scaffolds were made with ratios (w/w) of 90% PCL to 10% CaP and 80% PLC to 20% CaP.

2.4 Scanning Electron Microscopy (SEM) of Calibration Blocks and Scaffolds

Porogens and scaffold structures were prepared for characterization by SEM according to standard protocol[16]. Briefly, non-biological samples (porogens and empty scaffolds) were air-dried and sputter coated with Au/Pd for a period of 60-120 seconds depending on sample architecture. Biological samples (cell-seeded scaffolds) were fixed in 2.5 % aqueous gluteraldehyde for 1 hour at room temperature then overnight at 4ºC, dehydrated through graded alcohols, dried with a critical point dryer (SPI supplies). All samples were examined with a SEM (XL-30 Environmental SEM-FEG).

2.5 Mechanical Testing

Compression tests of solid rods made of PCL and PCL-CaP were performed on an Instron 5543 uniaxial testing system using 1KN load cell. Five specimens of each material were tested according to the guidelines specified in ASTM D695-02a[17]. In addition, compression testing was done on 600µm pore pure PCL scaffolds (n=6) at a compression rate of 1mm/min using the same system described above with a 100N load cell. Effective stress was computed based on the scaffold cross-sectional area. The ultimate compressive strength (UCS) as well as the compression modulus (CM) was calculated from the effective stress-strain diagrams, as previously described [17].

2.6 Cell Culture

The cytocompatibility of the scaffolds was assessed using human embryonic palatal mesenchymal cells (HEPM, ATCC, CRL-1486). These cells are routinely maintained in Eagles' minimum essential medium (MEM) with Earles' salts supplemented with 10% fetal bovine serum (Hyclone), 2.0mM L-glutamine, 1.0mM sodium pyruvate, 0.1 mM non-essential amino acids, and 1.5g/L sodium bicarbonate at 37°C in a 5% CO_2 incubator[16]. For cell culture studies, the scaffolds were sterilized with

70% ethanol for 1 hour, and washed 3 times with sterile phosphate buffered saline (PBS). The scaffolds were then incubated with a mixture of 30µg/ml collagen type I (BD Biosciences) and Matrigel™ (BD Biosciences, diluted 1:30) in MEM for 1 hour at 37°C to facilitate ECM protein adsorption and enhanced cellular attachment. Scaffolds were then seeded with a suspension of 1 million cells/ml overnight on an orbital shaker (Belly Dancer, Stovall). Following seeding, scaffolds were transferred to 24-well plates, allowed to equilibrate for 2 hours in the described cell culture medium and the initial level of cell seeding was assessed by the Alamar Blue (Biosource) assay[16]. In order to assess cell proliferation on the various scaffolds the Alamar Blue assay was performed again on the same samples at day 4 post-seeding. Subsequently, the samples were fixed in 10% buffered formalin (Fisher) for 1 hour at room temperature and stored in PBS at 4°C until cytological staining. For staining, the samples were washed once more with PBS and incubated with PBS containing 2µg/mL Hoechst 33258 (Bisbenzimide, Sigma), a nuclear stain.

2.7 Statistical analysis

All data are presented as means ± standard deviation (SD). For each experiment, the number of samples for each scaffold was 5. Statistical significance of Alamar Blue™ measurements was assessed by one way ANOVA with Tukey-Cramer post-tests for multiple comparisons, defining $P < 0.05$ as significant. Due to unequal variance, comparisons for the compressive testing were performed with one tailed t-test followed by the Welch correction for significantly different standard deviations.

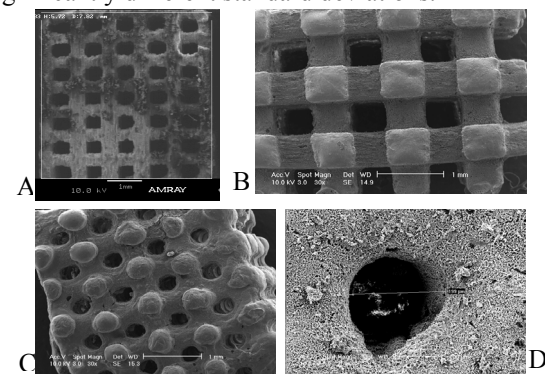


Figure 3: SEM of scaffolds, magnification and scale bar indicated in images. A: Thermoplastic porogen used for fabrication of 400µm scaffolds; B: PCL scaffold, 300µm pores. C: PCL-CaP composite scaffold, 600µm pores. D: PCL scaffold, single pore ~ 200µm.

3. Results and Discussion

3.1 Scaffold Fabrication

Using the thermoplastic porogens, we successfully generated PCL and PCL-CaP composite scaffolds by injection molding of molten polymer. Using these porogens, we then fabricated PCL and PCL-CaP composite scaffolds with nominal void sizes of 300, 400, and 600µm. The measured pore sizes of our scaffolds, as evaluated from SEM micrographs, conformed well to the predicted sizes, based on the designs of the porogens. The measured pore sizes of pure PCL scaffolds fabricated using thermoplastic porogens with void spaces of 400 and 600µm were 396 ± 40µm and 607±12µm (n=12). In addition, PCL scaffolds with 200µm pore diameters were fabricated.

We note that other SFF systems have been able to generate structures with features of 200µm or less using direct building methods with PCL[18-20]. Notably, using a solution casting approach, Vozzi et al.[20] developed a microsyringe deposition system capable of generating deposition line widths as low as 20µm using a 2.5% poly-L-lactic acid (PLLA)/20% PCL solution. However, the manufacturing process reported by these authors was limited to by the fact that it did not allow for the effective fabrication of macroscopic 3-D scaffolds.

The pore sizes in our scaffolds are comparable with some of the highest resolution SFF systems capable of fabricating 3-D macroscopic scaffolds reported in the literature. For example, Geng et al.[5] reported pore sizes of 200-500µm using a direct printing system with the polysaccharide chitosan. Darling and Sun[18] used precision extrusion deposition of computer-aided design (CAD) models to fabricate PCL scaffolds with pore sizes and strut widths of 200-300µm. Similarly, Zein et al.[19] used a fused deposition modeling of CAD models to fabricate PCL scaffolds with pore sizes of 160-700µm and deposited fibers of 260-370µm. All of these direct build SFF methods are limited by the fact that the manufacturing process must be re-configured for each material used. By contrast, in our approach the ubiquitous porogen is more versatile. The machine must be configured only once for the ubiquitous porogen which may then be filled with any biomaterial having a melting temperature below 75°C.

Taboas et al.[6] used an indirect thermoplastic porogen approach comparable to our process. However their process is complicated by the fact that an additional step of casting ceramic into the thermoplastic is required. Thus, upon dissolution of the thermoplastic, Taboas et al. injection-molded poly-lactic acid (PLA) into the ceramic to generate scaffolds with macropore sizes of 500µm and an internal microporous structure on the order of 50-100µm produced by salt leaching. Our process, which intrinsically is simpler than that of Taboas et al, is easily amenable to the introduction of salt leaching approaches; it would require an additional leaching step in aqueous medium following thermoplastic porogen removal.

3.2 Mechanical Testing

To test the mechanical properties of PCL-CaP composites we performed compression testing as described in Materials and Methods. As seen in Figure 4, the increase in CaP content of the composite results in a statistically significant increase in compressive modulus (CM) and ultimate compressive strength (UCS) of the samples ($p<0.002$, one-tailed t-test assuming unequal variances). This is particularly advantageous for making scaffolds for application in hard tissue engineering.

In addition to testing the mechanical properties of solid cylinders made of the diverse scaffold materials, we tested the compressive strength of pure PCL scaffolds with 600µm pore size. Scaffold stress-strain curves show

multiple failure points due to failure of the weakest strut, followed by collapse of the entire scaffold structure (Figure 5). Pure PCL scaffolds had UCS values of 2.77±0.26MPa and a CM of 44.0±3.2MPa. The UCS value is in line with reported values for trabecular bone from human mandibles ranging from 0.22~10.44MPa[21]. The small standard deviation (<10% coefficient of variation) for CM and UCS of the solid cylinders as well as 100% PCL scaffolds demonstrates the reproducibility of the mechanical properties achieved using this process.

3.3 Cell-Biomaterial Interactions

The cytocompatibility of PCL and PCL-CaP composite 3-D scaffolds was assessed using the Alamar Blue assay for cell metabolic activity/cell proliferation. HEPM cells growing on the scaffolds were visualized by fluorescent staining of cell nuclei, and SEM. Previously, we demonstrated that HEPM cells seeded onto the surface of thermoplastic-molded solid disks of calcium phosphate cement (CPC) attached and proliferated similarly to culture on the "gold standard" TCPS, indicating no significant limitations in cellular function due to potential residual thermoplastic components[15]. In the case of 3-D scaffolds of PCL and 80:20 PCL-CaP, HEPM cells were able to attach as evidenced by fluorescent nuclear staining with Hoechst 33258 (Figure 6). The images shown in Figure 6 indicate attachment onto the struts of both PCL (Figure 6A) and 80:20 PCL-CaP composite scaffolds (Figure 6B). Based on the Alamar Blue assay, the initial seeding efficacy was not significantly different for the materials used (data not shown). This similar level of HEPM cell attachment to all materials used was probably due to the fact that all scaffolds were pre-coated with a mixture of Matrigel™, a reconstituted ECM, and collagen type I solution. Without this coating, cellular attachment to the synthetic surfaces was minimal only (not shown).

Once attached, HEPM cells proliferated on all types of 3D scaffolds, as assessed from the Alamar Blue fluorescence data (Figure 7), with some differences between materials. The normalized cell proliferation data indicated statistically significant ($P<0.05$ by one-way ANOVA with Tukey-Cramer post-tests for multiple comparisons) differences between 80:20 PCL-CaP and PCL. The enhanced proliferation of the cells on the 80:20 PCL-CaP composite scaffolds compared to scaffolds made of pure PCL, indirectly inferred from the increase in a biochemical parameter (increase in Alamar Blue fluorescence, commensurate with enhanced cell proliferation) was corroborated qualitatively by the observed increase in the density of bisbenzimide nuclear staining following 4 days of post-seeding *in vitro* culture (Figure 8). We note that at this time point, cells were visibly growing both on the struts (Figure 8A and B), as well as in the interior pore structures of all scaffolds investigated (Figure 8C and D).

Further confirmation of cellular ingrowth into the scaffold center was performed after cutting the scaffolds into segments using a scalpel and visualizing the presence of cells on all interior surfaces by nuclear staining (Figure 8E). The histotypic morphology of HEPM cells on PCL and 80:20 PCL-CaP composite scaffolds was documented by SEM (Figure 9). As seen in Figure 9A the cells flatten on the rather smooth PCL surface. By contrast, on the 80:20 PCL-CaP the cells seem to form multilayer assemblies (Figure 9B). This enhanced morphology and multiplayer assembly on 80:20 PCL-CaP, as observed by SEM, further corroborates the increased density of nuclear staining (Figure 8) and significantly higher level of cell proliferation (Figure 7) compared to 100% PCL. In summary, these cytocompatibility tests clearly indicate that all porogen-based scaffolds when coated with suitable ECM proteins facilitate attachment and support proliferation of HEPM cells *in vitro*. In addition, our data suggest that the presence of CaP in the PCL-CaP composite enhances the proliferation and morphology of HEPM cells.

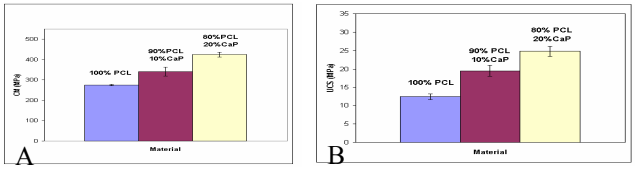


Figure 4: Compressive mechanical properties. A: Comparison of the compressive modulus of cylinders; B: Comparison of the ultimate compressive strength. Bars represent mean±standard deviation. Statistical analysis indicates that the material properties are significantly different for different concentrations of CaP.

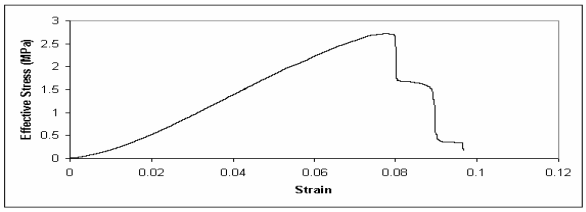


Figure 5: Typical effective stress-strain curve for 600µm 100% PCL scaffold.

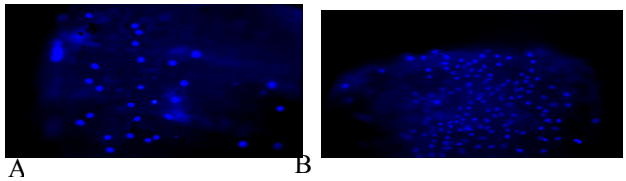


Figure 6: Bisbenzimide nuclear staining of adherent HEPM cells following 24 hours of orbital shaker seeding on PCL (A, 200x) and 80:20 PCL-CaP composite (B, 100x) scaffolds.

Our findings are in line with previous reports showing that PCL scaffolds fabricated using various manufacturing processes display good cytocompatibility *in vitro* [18,23] and excellent biocompatibility *in vivo*[7]. For example, Williams et al[7] used selective laser sintering (SLS) to fabricate PCL scaffolds which were then seeded with human gingival fibroblasts genetically modified to express bone morphogenetic protein-7 (BMP-

7) and implanted into subcutaneous pockets of immunocompromised mice. These scaffolds supported the development of new bone over a 4 week period, as evidenced by micro computed tomography detection of mineralized tissue[7]. Darling and Sun[18] reported the *in vitro* biocompatibility of precision extrusion deposition fabricated PCL scaffolds using rat cardiomyoblasts, however detailed analysis of cellular metabolism, proliferation, and morphology were not provided. Hutmacher et al.[23] used primary human fibroblasts and human osteoprogenitor cells to demonstrate the biocompatibility of PCL scaffolds fabricated by fused deposition modeling although the capacity of these scaffolds to induce bone formation was not addressed.

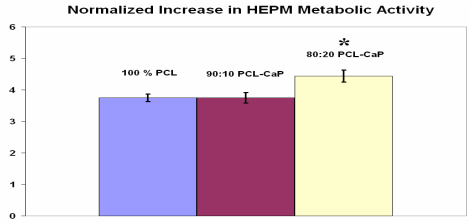


Figure 7: Normalized increase in Alamar Blue readings over the 4 day *in vitro* culture period following the initial 24 hour seeding period for 600µm pore size pure PCL, 90:10 and 80:20 PCL-CaP scaffolds. Y-error bars represent the standard deviation from the mean for each sample (n = 5).

Diverse scaffolds fabricated from CaP and CaP composites also display *in vitro*[24,25] and *in vivo*[26] biocompatibility. For example, Wang et al.[24] demonstrated the capacity of biomimetic nano-structured CaP scaffolds fabricated using gel lamination technology to support osteogenic differentiation, as evidenced by alkaline phosphatase expression. Xu et al.[25] used a murine osteoblast cell line to demonstrate biocompatibility of CaP-chitosan composites with amorphous architecture and pore sizes of 165-270 µm fabricated by preparing a water-soluble mannitol mixture with the CaP-chitosan, and subsequent removal of mannitol to create the pore structure. Amorphous poly(lactic-co-glycolic acid) PLGA-CaP scaffolds of various weight ratios fabricated by admixing PLGA microparticles into Ca-P cement and implanted into subcutaneous and cranial defects in rats, facilitated fibrovascular and bone tissue development over a 12 week period, respectively[26]. The primary advantage of SFF scaffolds compared to the amorphous CaP scaffolds mentioned above is the precisely generated structures that in turn allow for reproducible scaffold fabrication with control of mechanical properties.

4. Conclusion

The primary advantage of the porogen-based method described in this paper is the ability to use multiple biomaterials for injection molding with a single ubiquitous porogen. As another advantage, the indirect porogen technique can improve the resolution of our SFF system by at least 2-fold as compared to directly built scaffold fabrication. In this paper, we established a thermoplastic porogen-based injection molding manufacturing process and demonstrated efficient, reproducible fabrication of porous PCL and PCL-CaP composite scaffolds with pore sizes as small as 300 µm3. With their interconnected porous structure, these scaffolds will be suitable for several tissue engineering applications such as replacement of trabecular bone, etc. *In vitro* cytocompatibility has been demonstrated for both PCL and PCL-CaP scaffolds fabricated using the thermoplastic porogen methodology. Future work will focus on optimization of mechanical properties and investigating the ability to generate mineralized bone tissue constructs *in vitro* using PCL-CaP composite scaffolds seeded with mesenchymal stem cells.

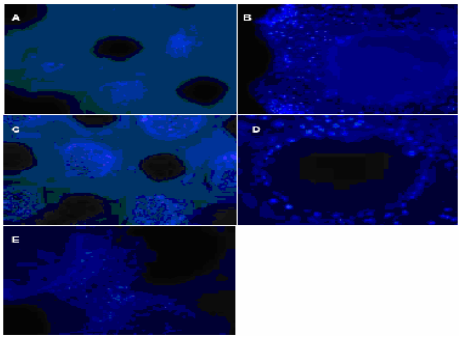


Figure 8: Bisbenzimide staining of HEPM cells cultured on PCL (A& B) and 80:20 PCL-CaP composite (C & D) scaffolds for 5 days. A: HEPM cells on the surface struts of a 600µm pore size PCL scaffold (50x); B: HEPM cells growing around and into a pore on the same scaffold imaged in A (100x); C: HEPM cells on the surface struts of a 600µm pore size 80:20 PCL-CaP composite scaffold (50x); D: HEPM cells colonizing a pore in the scaffold imaged in C (200x); E: HEPM cells growing on a strut from the scaffold center (100x).

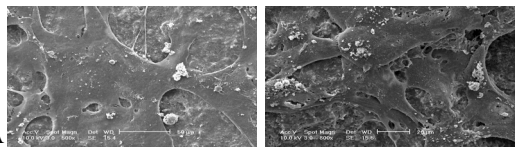


Figure 9: SEMs of HEPM cells cultured on 600 µm pore size PCL and 80:20 PCL-CaP scaffolds for 5 days. A: Flattened HEPM cells on PCL scaffold, Scale bar = 50 µm; B: Multilayered HEPM cells on 80:20 PCL-CaP scaffold, scale bar = 20 µm;

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