Published in final edited form as:

J Biomed Mater Res A. 2013 May ; 101(5): 1225–1236. doi:10.1002/jbm.a.34610.

Osteogenic differentiation of mesenchymal stem cells on demineralized and devitalized biodegradable polymer and extracellular matrix hybrid constructs

Richard A. Thibault, M.S.E., Antonios G. Mikos, Ph.D., and F. Kurtis Kasper, Ph.D.*

Department of Bioengineering, Rice University, MS-142, P.O. Box 1892, Houston, TX 77251

Abstract

Devitalization and demineralization processing of biodegradable polymer and extracellular matrix (ECM) hybrid constructs was explored for the effect on the retention of ECM components and construct osteogenicity. Hybrid constructs were generated by seeding osteogenically pre-differentiated rat mesenchymal stem cells (MSCs) onto electrospun poly(ϵ -caprolactone) fiber meshes and culturing in osteogenic medium for 12 or 16 days within a flow perfusion bioreactor to create an ECM coating. The resulting constructs were then either devitalized (using a freeze-thaw or a detergent technique), devitalized and demineralized, or left untreated, and subsequently characterized for DNA, glycosaminoglycan, collagen, and calcium content. The osteogenicity of each construct was investigated by culturing MSCs on the hybrid constructs within a flow perfusion bioreactor for 4, 8 and 12 days in osteogenic medium. Histological staining demonstrated that devitalization via the freeze-thaw method retained the thickest coating of ECM components within the constructs. Demineralization and devitalization processing of ECM coated constructs resulted in a decrease in their osteogenicity.

Keywords

Bone Tissue Engineering; Demineralization; Devitalization; Extracellular Matrix; Osteogenicity

Introduction

Craniofacial defects caused by trauma, disease, and congenital deformity generally can be repaired clinically using the current gold standard, autologous bone, if the defect is of limited size.[1] However, some patients present with defects involving a large volume that cannot be adequately repaired using their own bone tissue alone. To address this issue, allogeneic bone and demineralized bone matrix (DBM) have been employed to provide additional graft material for the repair of large defects.[2] However, these graft materials generally have had limited success at bone regeneration and integration with the surrounding bone at the defect site.[3–5] The limited success may be due to the devitalization; that is, the removal of a living cellular component, and sterilization processes required to generate allogeneic bone and DBM.[6] These processing steps have been shown to reduce the osteogenic and osteoinductive properties of the graft materials as compared to autologous bone.[1, 5]

The overarching goal of bone tissue engineering is to create an osteogenic and osteoinductive construct capable of promoting bone regeneration. Our laboratory has

*Corresponding Author: F. Kurtis Kasper, Ph.D. Faculty Fellow, Department of Bioengineering, Rice University, MS-142, P.O. Box 1892, Houston, TX 77251-1892, Phone: (713) 348-3027, Fax: (713) 348-4244, kasper@rice.edu.

developed a cell-generated extracellular matrix (ECM) coated polymer construct as a potential material for bone tissue engineering.[7, 8] Previous investigation into these constructs has determined that they are comprised of collagen type I and hydroxyapatite, along with other key cell-adhesive and regulatory proteins that are present within native bone.[9] In addition, pre-differentiated mesenchymal stem cells (MSCs) cultured on these constructs under static conditions and in medium lacking osteogenic supplements have displayed increased osteogenic differentiation when compared to MSCs cultured on materials lacking the ECM coating.[7] However, prior to the seeding of the MSCs, the constructs underwent a devitalization and sterilization process.[7, 9] Therefore, analogous to allogeneic bone grafts and DBM, these processed constructs may have a reduced osteogenic and osteoinductive potential as compared to untreated constructs.

The devitalization and demineralization techniques of cell-generated ECM coated constructs have been adapted from the processing of bone grafts and tissues.[10, 11] However, there has been little investigation into the effects of various devitalization methods on the composition of the cell-generated ECM coatings.[12–17] Accordingly, we investigated whether these modified bone graft processing techniques affected the retention of the ECM components and the osteogenic differentiation of MSCs cultured onto MSC-generated ECM coated constructs. Constructs were generated by seeding osteogenically pre-differentiated MSCs onto electrospun poly(ϵ -caprolactone) (PCL) fiber meshes and culturing them within a flow perfusion bioreactor for 12 days or 16 days in osteogenic medium. Subsequently, they were either i) sterilized, ii) sterilized and devitalized using a freeze-thaw method, iii) sterilized and devitalized using a detergent procedure, or iv) sterilized and devitalized and demineralized using a freeze-thaw method combined with an acid treatment. Each construct was characterized by measuring the amount of DNA, glycosaminoglycans (GAGs), collagen, and calcium within each construct to determine the extent of loss of biological and extracellular material due to processing. Scanning electron microscopy (SEM) and histological staining for GAGs, collagen, and calcium were also performed to determine the distribution of each component within the constructs. Furthermore, osteogenically pre-differentiated MSCs were seeded onto each cell-generated ECM coated construct and cultured in osteogenic medium within a flow perfusion bioreactor for 4, 8, and 12 days. The cellularity and mineralization of the construct, along with the alkaline phosphatase (ALP) activity of the MSCs was determined to elucidate the effects of processing on the osteogenic differentiation of cultured MSCs within the constructs. Additionally, SEM and histological staining was performed to determine ECM distribution throughout the constructs seeded with MSCs.

Methods and Materials

Materials

PCL with an inherent viscosity of 0.68 dL/g was purchased from DURECT Corporation (Pelham, AL). Ethylene oxide was obtained from Andersen Sterilizers, Inc. (Haw River, NC). Fischer 344 rats were purchased from Charles River Laboratories (Wilmington, MA). α -MEM was obtained from Invitrogen (Carlsbad, CA), while fetal bovine serum was purchased from Gemini Bio-Products (West Sacramento, CA). The PicoGreen assay kit was purchased from Molecular Probes (Eugene, OR). The Arsenazo III kit was purchased from Diagnostics Chemicals Limited (P.E.I., Canada). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Fabrication of PCL Fiber Meshes

The number average molecular weight (61000 ± 2500 Da) and the weight average molecular weight (88500 ± 2700 Da) of the PCL were determined via gel permeation chromatography

using polystyrene standards (n=6). The PCL was dissolved in a 5:1 (v/v) chloroform:methanol solution at 18% (w/w) prior to being electrospun to produce fiber mesh mats with a porosity of 87% and an average fiber diameter of approximately 10 μm , as previously described.[8] Disc-shaped meshes 8 mm in diameter and approximately 1 mm thick were prepared from the PCL mats using a biopsy punch, then subsequently sterilized by exposure to ethylene oxide for 14 hours, and pre-wetted using an ethanol gradient prior to cell seeding.

MSC Isolation

MSCs were harvested and pooled from the marrow of tibiae and femora of 4 male Fischer 344 rats (150 – 175 g) as previously described.[18] NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were strictly observed. The MSCs were cultured in complete osteogenic media (α -MEM, 10% fetal bovine serum, 10 mM β -glycerol-2-phosphate, 10 nM dexamethasone, 50 $\mu\text{g}/\text{mL}$ ascorbic acid, 50 $\mu\text{g}/\text{mL}$ gentamicin, 100 $\mu\text{g}/\text{mL}$ ampicillin, and 0.5 $\mu\text{g}/\text{mL}$ fungizone) for 7 days to pre-differentiate them along the osteogenic pathway.[18]

MSC Culture on PCL Fiber Meshes

Prior to cell seeding, pre-wetted PCL fiber meshes (n = 128) were transferred into complete osteogenic medium for 2 hours, press-fit into cassettes, and maintained briefly in an incubator. A quarter-million of the isolated MSCs in 200 μL of complete osteogenic medium were seeded onto each PCL fiber mesh, and the MSCs were allowed to adhere overnight in the incubator. Subsequently, the cassettes containing cell-seeded meshes were placed into flow perfusion bioreactors with a flow rate of 0.7 mL/min and 200 mL of complete osteogenic medium per bioreactor, which was exchanged every 3 days.[19] Sixty-four MSC-generated ECM coated constructs each were removed from the bioreactors at day 12 (PCL/ECM 12) and day 16 (PCL/ECM 16).

Construct Processing

The PCL/ECM 12 and PCL/ECM 16 constructs were processed using four different methods (n = 16) as described in Table 1. Constructs from each processed group (n = 3) were digested using Proteinase K to generate a sample solution and analyzed using the DNA, GAG, hydroxyproline, and calcium assays. One additional construct was fixed in 2.5% (v/v) glutaraldehyde for 45 minutes and cut in half for use in histological analysis and SEM.

Seeding of the ECM Coated Constructs with MSCs

Constructs from each of the groups listed in Table 1 (n = 12) were treated and seeded with MSCs in a similar manner as previously described in the section regarding MSC culture on PCL fiber meshes. Three constructs were removed from each group after 4, 8, and 12 days and placed in Millipore water. Constructs from each culture period underwent a repeated freeze-thaw procedure to generate a sample solution, which was later analyzed using the DNA, ALP, and calcium assays. To determine the calcium content, a volume of 1 N acetic acid equal to the volume of solution present in each sample tube was added to each sample solution and construct. The resulting 0.5 N acetic acid/sample solution was placed on a shaker table for 1 day at 100 rpm to dissolve calcium present in the construct. One construct was removed for each group from each culture period, fixed in 2.5% (v/v) glutaraldehyde for 45 minutes, cut in half, and retained for histological analysis and SEM.

Proteinase K Digestion

Proteinase K solution was prepared by adding 100 mg Proteinase K to 100 mL of Pepstatin A buffer and placing the solution within a 56°C water bath for 2 hours.[20] Constructs from

each of the processed groups ($n = 3$) were individually placed into 1 mL of Proteinase K solution and maintained in a 56°C water bath for 16 hours. Afterwards, the constructs and solution underwent three cycles of freezing and thawing followed by 10 minutes of ultrasonication.

Biochemical Assays

The amount of DNA present within the constructs was determined with the PicoGreen assay kit, using an excitation wavelength of 480 nm and measuring the emission at 520 nm, as previously described.[20] The amount of GAGs within each construct was determined with the 1,9-dimethyl-methylene blue (DMMB) assay, measuring absorbance at 520 nm as previously described.[20] The amount of collagen within each construct was determined with the hydroxyproline assay, measuring absorbance at 570 nm as previously described. [20] The measured hydroxyproline amounts were converted to collagen amounts by using a hydroxyproline to collagen ratio of 1:10.[21] The calcium amount was determined using an Arsenazo III kit, measuring absorbance at 650 nm as previously described.[7, 22] The ALP activity was determined using phosphatase substrate capsules and measuring absorbance at 405 nm as previously described.[7]

Scanning Electron Microscopy

After glutaraldehyde fixation, half of a construct was dehydrated in a gradient ethanol series, air dried in a laminar flow cell culture hood, lyophilized, and sputter coated with 20 nm of gold prior to imaging. Each construct was imaged using an FEI Quanta 400 ESEM FEG (FEI Company) at a magnification of 250X in the center of each construct.

Histological Stains

Sectioning of the processed constructs prior to seeding was performed by placing half of each construct into Histoprep overnight, followed by embedding and freezing into a block at -20°C. The blocks were cut into 5 µm-thick sections that were placed onto 4X UV adhesive slides (Electron Microscope Sciences) maintained at -20°C and adhered to the slide via a brief exposure to 345 nm UV light prior to defrosting of the slide at room temperature. Sections were stained with safranin O, picrosirius red, and alizarin red.[23–25] For the sectioning of the MSC-seeded constructs, half of a construct was placed into Histoprep overnight, embedded, and frozen into a block at -20°C. The blocks were cut into 5 µm-thick sections that were adhered to Superfrost Plus slides (VWR) and placed on a slide warmer set for 45°C for 1 day. Hematoxylin and eosin staining was performed on each of these sections. [26]

Statistical Analysis

Results are presented as means $\pm$ standard deviations. Statistical significance between all constructs was determined using Tukey's Honestly Significant Differences test with 95% confidence interval using JMP 9 software (SAS Institute).

Results

Amount of DNA, GAGs, Collagen, and Calcium within ECM Coated Constructs

As can be seen in Figure 1, the EO 12 and EO 16 constructs contained significantly more ($p < 0.05$) DNA as compared to the respective FT, Tri, and dM constructs. The processed constructs with the same ECM maturity contained similar amounts of DNA. The processed PCL/ECM 16 constructs contained significantly more ($p < 0.05$) DNA than the processed PCL/ECM 12 constructs. No significant difference was observed in GAG amounts between EO 12 and EO 16 as compared to the respective FT, Tri, and dM constructs (Figure 2).

However, there were significantly higher amounts of GAGs present within the PCL/ECM 16 constructs as compared to the respective PCL/ECM 12 constructs ($p < 0.05$). As illustrated in Figure 3, there was no significant difference in the amount of collagen contained within each construct between EO 12 and EO 16 as compared to the respective FT, Tri, and dM constructs. However, there were significantly higher amounts of collagen in the PCL/ECM 16 constructs as compared to the respective PCL/ECM 12 constructs, with the exception of the FT constructs ($p < 0.05$). No significant difference between the calcium amount within the processed PCL/ECM 12 constructs as compared to the respective sterilized constructs, EO 12, was found (Figure 4). Similarly, no significant difference in the calcium content of the processed PCL/ECM 16 constructs was observed as compared to the respective sterilized EO 16 constructs, with the exception of the dM 16 constructs, which presented significantly less calcium than the EO 16 constructs ($p < 0.05$).

Distribution of ECM Components within the Processed Constructs

The SEM micrographs in Figure 5 show the presence of ECM at the surface of the EO 12, FT 12, and Tri 12 constructs, with minimal amounts of ECM present on the dM 12 constructs. The ECM component present at the surface of the EO 16, FT 16, and Tri 16 constructs appeared to have few void spaces, relative to the ECM at the surface of the dM 16 construct.

Figures 6i(A) and 6ii(A) demonstrate that an ECM component containing GAGs, as indicated by safranin O staining, and collagen, as indicated by picrosirius red staining, was localized primarily within the top 100 microns of the EO 12 constructs. Similarly, an ECM component containing GAGs and collagen was present at the top of the FT 12 constructs (Figures 6i(B) and 6ii(B)), although the thickness of the ECM layer appeared to be less than in the EO 12 constructs. However, negligible amounts of ECM were observed in the Tri 12 and dM 12 constructs (Figures 6i(C–D) and 6ii(C–D)). In contrast, the EO 16 construct demonstrated an ECM component containing GAGs and collagen located throughout the thickness of the construct (Figures 6i(E) and 6ii(E)). An ECM component containing GAGs was observed at the surface of the FT 16 construct, with some pockets of ECM containing GAGs present within the interior of the construct (Figure 6i(F)). The FT 16 construct was also seen to have ECM containing collagen present throughout the interior of the construct (Figure 6ii(F)). Both the Tri 16 and dM 16 constructs had a thin coating of ECM containing GAGs and collagen at the surface of the constructs (Figures 6i(G–H) and 6ii(G–H)). ECM containing calcium, as indicated by alizarin red staining, generally was localized in the same areas as the collagen component of each construct (Supplemental Figure 1).

Cellularity of Processed Constructs Seeded with MSCs

No significant difference was observed in cell number in the EO 12, FT 12, and dM 12 constructs at day 12 (Figure 7). However, there was a significant difference ($p < 0.05$) in cell number between Tri 12 and EO 12 constructs at day 12. The dM 12 constructs had a significantly lower ($p < 0.05$) amount of cells present as compared to FT 12 and Tri 12 at day 8. Among the EO 16, FT 16, and Tri 16 constructs, there was no significant difference in cell number at any culture period. However, the dM 16 constructs had a significantly higher ($p < 0.05$) cell number as compared to the EO 16, FT 16, and Tri 16 constructs at day 12. A significantly higher ($p < 0.05$) number of cells was seen within EO 12, FT 12, and Tri 12 constructs when compared to the EO 16, FT 16, and Tri 16 constructs, respectively, at day 8. There was no significant difference in the cell number between dM 12 and dM 16 constructs at days 4, 8, and 12.

Alkaline Phosphatase Activity of MSCs Seeded on Processed Constructs

The alkaline phosphatase activity of the MSCs seeded onto the ECM coated constructs is presented in Figure 8. There was no significant difference within the EO 12, FT 12, Tri 12, and dM 12 constructs at days 4, 8, and 12. EO 16 constructs demonstrated a significantly higher ($p<0.05$) ALP activity as compared to the dM 16 constructs at day 8. Comparing between the EO 16, FT 16, and Tri 16 constructs, the ALP activity of the FT 16 constructs was significantly higher than the other groups at day 12 ($p<0.05$). The EO 16 constructs had a significantly higher ($p<0.05$) ALP activity when compared to EO 12 constructs at days 4 and 12. Similarly, the FT 16 constructs had a significantly higher ($p<0.05$) ALP activity than FT 12 constructs at day 12.

Calcium Deposited on Processed Constructs Seeded with MSCs

Figure 9 demonstrates that there was no significant difference in calcium deposition between the EO 12, FT 12, and Tri 12 constructs at any particular time point. However, the Tri 12 constructs had a significantly higher ($p<0.05$) amount of calcium as compared to dM 12 constructs at day 12. The EO 16 constructs had a significantly higher ($p<0.05$) amount of calcium when compared to either FT 16 or dM 16 constructs at days 8 and 12. Nevertheless, there was no significant difference between the EO 12, FT 12, Tri 12, and dM 12 constructs when compared to the EO 16, FT 16, Tri 16, and dM 16 constructs, respectively, at any time point.

Extracellular Matrix Distribution within Processed Constructs Seeded with MSCs

The scanning electron micrographs in Figure 10 show an ECM coating on the surface of each of the constructs, with only the Tri 12 construct lacking a full coating. In addition, the EO 16 construct appeared to have a layer of ECM overlying another layer of ECM that is present at the top surface of construct (Figure 10(E)).

Figure 11i(A–C) demonstrates that the ECM component of the EO 12 construct increased from a thin surface layer at day 4 to be present throughout the construct by day 12. Additionally, the EO 16 construct at day 12 appears to contain a thin layer of ECM extending above the ECM that is present throughout the construct (Figure 11i(F)). Figure 11ii(A–C) shows that the FT 12 construct had an ECM component located primarily at the surface that increased in thickness with time. Meanwhile, the FT 16 construct was seen to have an ECM component present throughout the construct from day 4 through day 12 (Figure 11ii(D–F)). Both the Tri 12 and Tri 16 constructs had an ECM component primarily at the surface of each construct by day 8, which appeared to decrease in thickness by day 12 (Supplemental Figure 2i). In contrast, the dM 12 construct at day 8 had an ECM component at the surface of the construct that increased in thickness by day 12 (Supplemental Figure 2ii). The dM 16 constructs demonstrated a sparse ECM component at days 4 and 8 that increased considerably by day 12 (Supplemental Figure 2ii).

Discussion

The goal of the present study was to determine the effects of devitalization and demineralization processing of flow perfusion cell-generated ECM coated constructs on the retention of ECM components and construct osteogenicity. Characterization of the constructs explored the amount and distribution of DNA, GAGs, collagen, and calcium, while MSC-seeding of the constructs investigated cellular proliferation using DNA amounts and osteogenic differentiation using the well-established markers of ALP activity and calcium content.[27, 28]

The characterization of the constructs demonstrated that all of the devitalization and demineralization techniques tested were successful in removing cells and calcium-bearing minerals. Specifically, the amount of DNA present in the EO 12 and EO 16 constructs was significantly higher than those in any of the respective processed constructs. Each processed construct contained similar amounts of DNA, suggesting that no devitalization technique was successful in removing all of the nuclear material. However, if complete removal of the nuclear material was desired, treatment of the constructs with DNases and RNases may potentially be a solution.[29, 30] Negligible levels of calcium were observed for both of the dM 12 and dM 16 constructs after demineralization, demonstrating that the 2% EDTA treatment for 2 hours is sufficient to remove the calcium deposits. In addition to the successful devitalization and demineralization, no significant difference in the amounts of GAGs, collagen, and calcium was measured in each of the FT, Tri, and dM constructs when compared to the respective EO constructs. The implications are that the devitalization and demineralization process had minimal impact on the amount of GAGs, collagen, and calcium present in the constructs.

However, visual examination of the scanning electron micrographs in Figure 5 provides additional information, with lower amounts of ECM observed on the dM 12 constructs as compared to the EO 12, FT 12, and Tri 12 constructs and markedly lower amounts of ECM observed on the dM 16 constructs as compared to the EO 16, FT 16, and Tri 16 constructs. Similarly, the histological sections show a drastic decrease in the thickness of the GAG, collagen, and calcium coatings within the constructs after devitalization and demineralization. The discrepancy between the quantitative and qualitative analyses may be a result of the methodology used to gather the images. The scanning electron micrographs obtained showed only the ECM present on the surface of the constructs and do not show any ECM that may be present within the interior of the constructs. With regard to the histological images, the processing performed to create the FT, Tri, and dM constructs may have allowed for ECM loss to occur while generating fixed and embedded samples for sectioning.

Further study of the histological images illustrates that there are differences in the thickness of the GAG coating for both PCL/ECM 12 and PCL/ECM 16 constructs. The EO constructs have a thicker coating of GAGs as compared to the FT constructs, which in turn have a thicker coating of GAGs than either the Tri or dM constructs. A similar loss in collagen thickness is observed in the picrosirius red staining of the FT, Tri, and dM constructs as compared to the EO constructs. Nevertheless, the FT constructs contain a thicker coating of collagen than either the Tri or dM constructs. In each treatment, the processed constructs are either exposed to water or ammonium hydroxide in order to cause cell lysis, but these neither destroy nor remove the GAGs or collagen from the ECM.[11, 31] However, exposure of the PCL/ECM constructs to freezing and thawing, ultrasonication, or continuous stirring can disrupt the ECM, while Triton X-100 exposure has been shown to remove GAGs in addition to disrupting the ECM.[11] Thus, the observation of a thinner coating of GAGs and collagen in the Tri construct as compared to the EO and FT constructs can be explained by the likely removal of GAGs by the Triton X-100 treatment. The loss of GAGs and collagen observed in the dM constructs as compared to the FT constructs may also be explained by the additional demineralization step. The dM constructs underwent the same devitalization steps as the FT constructs, but they were also soaked in a 2% EDTA solution that was continuously stirred for 2 hours. The extra time the dM constructs were agitated could potentially disrupt the ECM to a greater extent, resulting in a thinner coating of GAGs and collagen.

In a similar trend to the collagen distribution, the alizarin red staining of the FT, Tri, and dM constructs demonstrated that there was a thinner coating of calcium present as compared to

the EO constructs, but the FT constructs contained a thicker coating of calcium than the Tri or dM constructs. The loss in calcium thickness in each of the processed constructs may be explained by the initiation and preferential deposition of calcium minerals along the collagen fibers typical in bone formation.[32, 33] The devitalization and demineralization techniques likely removed some of the collagen fibers and thus the calcium minerals bound to the collagen fibers might also have been removed. The end result is a similar trend between the calcium staining and collagen staining.

The MSC culture on the constructs demonstrated that cellular proliferation was similar on all of the PCL/ECM 12 constructs, but the dM 16 constructs had a significantly higher number of cells as compared to the EO 16, FT 16, and Tri 16 constructs. Examining the scanning electron micrographs of each of the constructs seen in Figure 5, each of the PCL/ECM 12 constructs had void spaces allowing access to the interior of the construct. However, among the PCL/ECM 16 constructs, the EO 16 constructs had minimal void space, the FT 16 and Tri 16 constructs had more voids, while a relatively large amount of void space was observed in the dM 16 constructs.

The void spaces observed within all of these cell-generated ECM coated constructs may potentially also be caused by the various devitalization and demineralization conditions. Each of the treatments used within this study has been shown to disrupt the ECM.[11] In the freeze-thaw devitalization method, numerous small ice crystals are created by the rapid freezing of water that leave gaps where the cells were present and ice crystals had formed.[34] With the detergent method, the lipids present in the cell membrane would be dissociated, creating spaces where cells once were present.[35] In the case of the demineralization procedure, some proteins and proteoglycans present within the ECM coating may have been solubilized or disrupted by the chelating agent, EDTA.[36] In addition, the agitation during the demineralization procedure generates convective forces throughout the construct.[37] Both EDTA and agitation would cause some loss of ECM components from the constructs, thus leaving gaps in the ECM coating.

With the presence of void spaces, the MSCs seeded onto the PCL/ECM 12 constructs and dM 16 constructs may have been able to infiltrate into the interior and proliferate. Indeed, the ability of MSCs to infiltrate the interior of the constructs is supported by similar cell numbers on dM 16 constructs at day 12 as compared to the PCL/ECM 12 constructs at day 12. Further support is demonstrated in the histological staining of the EO 12, FT 12, dM 12, and dM 16 constructs, where ECM can be observed within the interior of the constructs.

MSCs seeded onto Tri 12 constructs were also able to access the interior of the construct and generated an ECM coating at the top of the construct by day 8, but by day 12, the ECM coating had largely disappeared. Potentially, there may have been some remnants of Triton X-100 left within the constructs that could cause cell lysis. Consequently, proteases may have been released, which would have digested the ECM, resulting in a thin coating of ECM by day 12.

Meanwhile the histological staining of the EO 16 constructs demonstrate that the MSCs may have had difficulty accessing the interior of the construct and consequently generated a layer of ECM above the primary ECM coating. However, the FT 16 constructs allowed some MSC access into the interior of the construct and had increases in ECM coating at the top and bottom of the construct with minimal increase in matrix in the interior of the constructs. Similarly, the Tri 16 constructs allowed MSCs access and had an ECM coating present at the top of the construct at day 8, but this ECM coating in the Tri 16 constructs later disappeared by day 12, as with the Tri 12 constructs.

The cell proliferation data also show that the dM 12 constructs had a significantly lower number of cells present at day 8 when compared to the EO 12, FT 12, and Tri 12 constructs. The lower number of cells may be a result of the dM 12 construct having lost a greater thickness of GAGs and collagen than the other PCL/ECM 12 constructs. With this loss of ECM components, the MSCs may have had difficulty proliferating until they deposited sufficient ECM within the construct.

The ALP activity of the seeded MSCs demonstrates that there was no significant difference in activity between any of the PCL/ECM 12 constructs, which suggests that the processing techniques did not remove any of the osteogenic components from the deposited ECM. However, the EO 16 construct had a significantly higher ALP activity as compared to dM 16 and Tri 16 at day 8 and day 12, respectively. Here, the demineralization and detergent processing may have removed an osteogenic component present in the unprocessed constructs, since this effect is not seen in the FT 16 construct. In fact, the FT 16 constructs demonstrated a significantly higher ALP activity at day 12 as compared to the EO 16, Tri 16, and dM 16 constructs. This difference in ALP activity may be due to the combination of higher amounts of ECM components as compared to the Tri 16 and dM 16 constructs in conjunction with greater access to the interior of the construct as compared to the EO 16 constructs, which may have prolonged the osteogenic differentiation of the MSCs. In addition, since the difference in ALP activity between the processed constructs is only seen in the PCL/ECM 16 constructs, there may be an important osteogenic component present in the PCL/ECM 16 not found in the PCL/ECM 12. Further support for the presence of an osteogenic component can be seen by the significant difference in ALP activity between the EO 12 and EO 16 constructs at day 12 and between the FT 12 and FT 16 constructs at day 12.

The calcium data illustrate that there was no significant difference in the calcium deposition between the EO 12, FT 12, Tri 12, and dM 12 constructs and the similarity between the constructs may be explained by the similarity between their ECM coatings seen in the scanning electron micrographs and the histological images. Indeed, the cell proliferation and ALP activity data demonstrates that there were no significant differences between the constructs.

The significant difference observed between the calcium deposition in FT 16 and EO 16 constructs may be related to the ALP activity. ALP activity is an early osteogenic differentiation marker and calcium deposition is a late stage marker. Since the MSCs seeded onto FT 16 constructs demonstrated a significantly higher ALP activity than MSCs seeded onto EO 16 constructs, the MSCs may not have differentiated into osteoblasts by day 12.

There was a significant difference in calcium deposited for the dM 16 constructs at days 8 and 12 as compared to the EO 16 constructs, respectively. Certainly, the dM constructs begin with very little mineral component as compared to any of the other constructs. However, combining the calcium results with the ALP activity data, the difference may be explained also by a lower osteogenicity due to the loss of the mineral component. Calcium phosphate minerals present on a construct can adsorb proteins, such as growth factors from the surrounding medium and thus enhance the osteoinductivity of a mineralized construct. [38] In addition, it has been shown that a mineralized surface can increase ALP activity in seeded cells.[39]

Interestingly, there were no significant differences in calcium contents of the PCL/ECM 12 constructs and their respective PCL/ECM 16 constructs, which may suggest that both the 12 day and 16 day ECM coatings are equally osteogenic. However, it may also be a result of

minimal amounts of ECM present in the PCL/ECM 12 constructs and the lack of access into the interior of the construct for the PCL/ECM 16 constructs.

Conclusions

This study explored the effect of devitalization and demineralization techniques on the retention of ECM components within cell-generated ECM coated constructs and the osteogenic differentiation of MSCs seeded onto these constructs. Of all the processing techniques examined, histological analysis demonstrated that the freeze-thaw method contained the thickest coating of GAGs, collagen, and calcium within the constructs. In addition, the loss of minerals from the ECM coated constructs via the demineralization and devitalization process resulted in a reduced osteogenicity of the constructs. Moreover, each of the devitalization and demineralization techniques generated void spaces in the ECM coating, which generally increased with the number of processing steps. Overall, the methods applied in the processing of cell-generated ECM coated constructs were found to impact the composition and associated osteogenicity of the constructs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R01 AR057083). R.A.T. also acknowledges the Ruth L. Kirschstein National Research Service Award (F31 AR055874) from the National Institute of Arthritis and Musculoskeletal and Skin Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Arthritis and Musculoskeletal and Skin Diseases or the National Institutes of Health.

References

1. Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. *BMC Med.* 2011; 9:66–76. [PubMed: 21627784]
2. Eppley BL, Pietrzak WS, Blanton MW. Allograft and alloplastic bone substitutes: a review of science and technology for the craniomaxillofacial surgeon. *J Craniofac Surg.* 2005; 16(6):981–989. [PubMed: 16327544]
3. Mulliken JB, Glowacki J, Kaban LB, Folkman J, Murray JE. Use of demineralized allogeneic bone implants for the correction of maxillocraniofacial deformities. *Ann Surg.* 1981; 194(3):366–372. [PubMed: 7023397]
4. Piattelli A, Scarano A, Corigliano M, Piattelli M. Comparison of bone regeneration with the use of mineralized and demineralized freeze-dried bone allografts: a histological and histochemical study in man. *Biomaterials.* 1996; 17(11):1127–1131. [PubMed: 8718974]
5. Zimmermann G, Moghaddam A. Allograft bone matrix versus synthetic bone graft substitutes. *Injury.* 2011; 42(Suppl 2):S16–21. [PubMed: 21889142]
6. Finkemeier CG. Bone-grafting and bone-graft substitutes. *J Bone Joint Surg Am.* 2002; 84-A(3):454–464. [PubMed: 11886919]
7. Thibault RA, Scott Baggett L, Mikos AG, Kasper FK. Osteogenic differentiation of mesenchymal stem cells on pregenerated extracellular matrix scaffolds in the absence of osteogenic cell culture supplements. *Tissue Eng Part A.* 2010; 16(2):431–440. [PubMed: 19863274]
8. Pham QP, Sharma U, Mikos AG. Electrospun poly(epsilon-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: characterization of scaffolds and measurement of cellular infiltration. *Biomacromolecules.* 2006; 7(10):2796–2805. [PubMed: 17025355]
9. Thibault RA, Mikos AG, Kasper FK. Protein and mineral composition of osteogenic extracellular matrix constructs generated with a flow perfusion bioreactor. *Biomacromolecules.* 2011; 12(12):4204–4212. [PubMed: 22040097]

10. Bowman SM, Zeind J, Gibson LJ, Hayes WC, McMahon TA. The tensile behavior of demineralized bovine cortical bone. *J Biomech.* 1996; 29(11):1497–1501. [PubMed: 8894931]
11. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. *Biomaterials.* 2011; 32(12):3233–3243. [PubMed: 21296410]
12. Lu H, Hoshiba T, Kawazoe N, Chen G. Autologous extracellular matrix scaffolds for tissue engineering. *Biomaterials.* 2011; 32(10):2489–2499. [PubMed: 21211834]
13. Tour G, Wendel M, Tcacencu I. Cell-derived matrix enhances osteogenic properties of hydroxyapatite. *Tissue Eng Part A.* 2011; 17(1–2):127–137. [PubMed: 20695777]
14. Datta N, Holtorf HL, Sikavitsas VI, Jansen JA, Mikos AG. Effect of bone extracellular matrix synthesized in vitro on the osteoblastic differentiation of marrow stromal cells. *Biomaterials.* 2005; 26(9):971–977. [PubMed: 15369685]
15. Kruyt MC, de Bruijn JD, Wilson CE, Oner FC, van Blitterswijk CA, Verbout AJ, Dhert WJ. Viable osteogenic cells are obligatory for tissue-engineered ectopic bone formation in goats. *Tissue Eng.* 2003; 9(2):327–336. [PubMed: 12740095]
16. Dahl SL, Koh J, Prabhakar V, Niklason LE. Decellularized native and engineered arterial scaffolds for transplantation. *Cell Transplant.* 2003; 12(6):659–666. [PubMed: 14579934]
17. Gui L, Chan SA, Breuer CK, Niklason LE. Novel utilization of serum in tissue decellularization. *Tissue Eng Part C Methods.* 2010; 16(2):173–184. [PubMed: 19419244]
18. Holtorf HL, Jansen JA, Mikos AG. Flow perfusion culture induces the osteoblastic differentiation of marrow stroma cell-scaffold constructs in the absence of dexamethasone. *J Biomed Mater Res A.* 2005; 72(3):326–334. [PubMed: 15657936]
19. Bancroft GN, Sikavitsas VI, Mikos AG. Design of a flow perfusion bioreactor system for bone tissue-engineering applications. *Tissue Eng.* 2003; 9(3):549–554. [PubMed: 12857422]
20. Liao J, Guo X, Nelson D, Kasper FK, Mikos AG. Modulation of osteogenic properties of biodegradable polymer/extracellular matrix scaffolds generated with a flow perfusion bioreactor. *Acta Biomater.* 2010; 6(7):2386–2393. [PubMed: 20080214]
21. Stegemann H, Stalder K. Determination of hydroxyproline. *Clin Chim Acta.* 1967; 18(2):267–273. [PubMed: 4864804]
22. Guo X, Park H, Liu G, Liu W, Cao Y, Tabata Y, Kasper FK, Mikos AG. In vitro generation of an osteochondral construct using injectable hydrogel composites encapsulating rabbit marrow mesenchymal stem cells. *Biomaterials.* 2009; 30(14):2741–2752. [PubMed: 19232711]
23. Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J.* 1979; 11(4):447–455. [PubMed: 91593]
24. Aaron JE, Shore PA, Shore RC, Beneton M, Kanis JA. Trabecular architecture in women and men of similar bone mass with and without vertebral fracture: II. Three-dimensional histology. *Bone.* 2000; 27(2):277–282. [PubMed: 10913922]
25. Kiviranta I, Jurvelin J, Tammi M, Saamanen AM, Helminen HJ. Microspectrophotometric quantitation of glycosaminoglycans in articular cartilage sections stained with Safranin O. *Histochemistry.* 1985; 82(3):249–255. [PubMed: 2581923]
26. Ferdous Z, Wei VM, Iozzo R, Hook M, Grande-Allen KJ. Decorin-transforming growth factor-interaction regulates matrix organization and mechanical characteristics of three-dimensional collagen matrices. *J Biol Chem.* 2007; 282(49):35887–35898. [PubMed: 17942398]
27. Li IW, Cheifetz S, McCulloch CA, Sampath KT, Sodek J. Effects of osteogenic protein-1 (OP-1, BMP-7) on bone matrix protein expression by fetal rat calvarial cells are differentiation stage specific. *J Cell Physiol.* 1996; 169(1):115–125. [PubMed: 8841428]
28. Lian JB, Stein GS. The developmental stages of osteoblast growth and differentiation exhibit selective responses of genes to growth factors (TGF beta 1) and hormones (vitamin D and glucocorticoids). *J Oral Implantol.* 1993; 19(2):95–105. discussion 136–107. [PubMed: 8246305]
29. Yang B, Zhang Y, Zhou L, Sun Z, Zheng J, Chen Y, Dai Y. Development of a porcine bladder acellular matrix with well-preserved extracellular bioactive factors for tissue engineering. *Tissue Eng Part C Methods.* 2010; 16(5):1201–1211. [PubMed: 20170425]

30. Bader A, Schilling T, Teebken OE, Brandes G, Herden T, Steinhoff G, Haverich A. Tissue engineering of heart valves--human endothelial cell seeding of detergent acellularized porcine valves. *Eur J Cardiothorac Surg.* 1998; 14(3):279–284. [PubMed: 9761438]
31. Grunert M, Dombrowski C, Sadasivam M, Manton K, Cool SM, Nurcombe V. Isolation of a native osteoblast matrix with a specific affinity for BMP2. *J Mol Histol.* 2007; 38(5):393–404. [PubMed: 17682830]
32. Bilezikian, JP.; Raisz, LG.; Rodan, GA., editors. *Principles of Bone Biology.* 2. Vol. 1. Academic Press; San Diego, USA: 2002. p. 1
33. Tampieri A, Sprio S, Sandri M, Valentini F. Mimicking natural bio-mineralization processes: a new tool for osteochondral scaffold development. *Trends Biotechnol.* 2011; 29(10):526–535. [PubMed: 21645938]
34. Meryman HT. Physical limitations of the rapid freezing method. *Proc R Soc Lond B Biol Sci.* 1957; 147(929):452–459. [PubMed: 13494458]
35. Helenius A, Simons K. The binding of detergents to lipophilic and hydrophilic proteins. *J Biol Chem.* 1972; 247(11):3656–3661. [PubMed: 4113126]
36. Maurer P, Hohenester E. Structural and functional aspects of calcium binding in extracellular matrix proteins. *Matrix Biol.* 1997; 15(8–9):569–580. discussion 581. [PubMed: 9138289]
37. Sikavitsas VI, Bancroft GN, Mikos AG. Formation of three-dimensional cell/polymer constructs for bone tissue engineering in a spinner flask and a rotating wall vessel bioreactor. *J Biomed Mater Res.* 2002; 62(1):136–148. [PubMed: 12124795]
38. Kretlow JD, Mikos AG. Review: mineralization of synthetic polymer scaffolds for bone tissue engineering. *Tissue Eng.* 2007; 13(5):927–938. [PubMed: 17430090]
39. Salgado AJ, Figueiredo JE, Coutinho OP, Reis RL. Biological response to pre-mineralized starch based scaffolds for bone tissue engineering. *J Mater Sci Mater Med.* 2005; 16(3):267–275. [PubMed: 15744619]

Figure 1.

DNA content within each of the different processed constructs. Groups not connected by the same letter are statistically different ($p < 0.05$). Data are expressed as means $\pm$ standard deviation for $n=3$.

Figure 2.

Glycosaminoglycan content within each of the different processed constructs. Groups not connected by the same letter are statistically different ($p < 0.05$). Data are expressed as means $\pm$ standard deviation for $n=3$.

Figure 3. Collagen content within each of the different processed constructs. Groups not connected by the same letter are statistically different ($p < 0.05$). Data are expressed as means $\pm$ standard deviation for $n=3$.

Figure 4. Calcium content within each of the different processed constructs. Groups not connected by the same letter are statistically different ($p < 0.05$). Data are expressed as means $\pm$ standard deviation for $n=3$.

Figure 5. Scanning electron micrographs of processed constructs at 250X magnification. A) EO 12, B) FT 12, C) Tri 12, D) dM 12, E) EO 16, F) FT 16, G) Tri 16, H) dM 16.

Figure 6. Histological images of processed constructs at 5X magnification. Constructs were cut into 5 μm sections and stained with i) Safranin O and ii) Picrosirius Red. A) EO 12, B) FT 12, C) Tri 12, D) dM 12, E) EO 16, F) FT 16, G) Tri 16, H) dM 16.

Figure 7. Cellularity of reseeded constructs at day 4, day 8, and day 12. Groups not connected by the same letter are statistically different ($p < 0.05$). Data are expressed as means $\pm$ standard deviation for $n=3$.

Figure 8.

Alkaline phosphatase activity of reseeded constructs at day 4, day 8, and day 12. Groups not connected by the same letter are statistically different ($p < 0.05$). Data are expressed as means $\pm$ standard deviation for $n=3$.

Figure 9.

Calcium content present on each reseeded constructs at day 4, day 8, and day 12. Groups not connected by the same letter are statistically different ($p < 0.05$). Data are expressed as means $\pm$ standard deviation for $n=3$.

Figure 10.

Scanning electron micrographs of reseeded processed constructs at day 12 at 250X magnification. A) EO 12, B) FT 12, C) Tri 12, D) dM 12, E) EO 16, F) FT 16, G) Tri 16, H) dM 16.

Figure 11.

Histological images of the reseeded processed constructs at 5X magnification at day 4, day 8, and day 12. Constructs were cut into 5 μm sections and stained with H & E. i) EO constructs and ii) FT constructs. A) day 12 ECM 4 day time point, B) day 12 ECM 8 day time point, C) day 12 ECM 12 day time point, D) day 16 ECM 4 day time point, E) day 16 ECM 8 day time point, F) day 16 ECM 12 day time point.

Table 1

The processing methods and the techniques used to generate devitalized and demineralized constructs.

Processing Method	Technique	Initial Construct	Resulting Construct
Control	1 Exposure to ethylene oxide for 14 hours	PCL/ECM 12	EO 12
		PCL/ECM 16	EO 16
Freeze-Thaw Devitalization and Sterilization	1 Place in 1 mL of Millipore filtered water	PCL/ECM 12	FT 12
	2 3 cycles of freezing at -196°C followed by thawing at 37°C	PCL/ECM 16	FT 16
	3 10 minutes of ultrasonication		
	4 2 rinses with Millipore filtered water		
	5 Air dry		
	6 Exposure to ethylene oxide for 14 hours		
Detergent Devitalization and Sterilization	1 Place in 1 mL of 0.5% v/v Triton-X 100 and 0.05% v/v NH_4OH	PCL/ECM 12	Tri 12
	2 Shake at 100 rpm for 1 hour at room temperature	PCL/ECM 16	Tri 16
	3 3 rinses with Millipore filtered water		
	4 Air dry		
	5 Exposure to ethylene oxide for 14 hours		
Demineralization and Devitalization and Sterilization	1 Place in 1 mL of Millipore filtered water	PCL/ECM 12	dM 12
	2 3 cycles of freezing at -196°C followed by thawing at 37°C	PCL/ECM 16	dM 16
	3 10 minutes of ultrasonication		
	4 Place in 40 mL of 2% w/v ethylenediaminetetraacetic acid (EDTA)		
	5 Stir at 72 rpm for 2 hours		
	6 2 rinses with Millipore filtered water		
	7 Exposure to ethylene oxide for 14 hours		