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Remineralization of Demineralized Bone Matrix (DBM) via Alternating Solution Immersion (ASI)

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Abstract

In order to achieve successful clinical outcomes, biomaterials used for bone grafts must possess a number of traits including biocompatibility and osteoconductivity. These materials must also demonstrate appropriate mechanical stability to withstand handling as well as support potentially significant stresses at the implant site. Synthetic and natural polymer scaffolds used for bone tissue engineering (BTE) often lack necessary mechanical properties. Our goal was to internally mineralize natural collagenous matrix, thereby increasing mechanical properties of the material to useful levels.

Published methods for intrafibrillar collagen mineralization were applied to clinically relevant-sized constructs but did not successfully deposit mineral in the interior of the constructs. To address this limitation, we developed a new technique for the remineralization of demineralized bone matrix (DBM) based on alternating solution immersion, or ASI.

Mineral was removed from equine bone specimens, leaving behind a demineralized bone matrix (DBM). This matrix provides a framework for the nucleation and growth of a replacement mineral phase. Plain film radiography and microcomputed tomography (microCT) indicated accumulation of mineral within the DBM, and mechanical testing (3 point bending and compression) revealed a significant increase in stiffness between the DBM and the remineralized bone matrix (RBM). We believe this remineralization process will be useful in the preparation of stiff and strong allografts for clinical application.

Keywords

Mineralization; Bone graft; Tissue engineering; Collagen; Matrix

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1.1 Introduction

Up to 10% of fractures in the US each year suffer from insufficient or delayed healing and require some form of exogenous treatment (Praemer et al., 1992). Autografts are the current clinical gold standard for treatment, but they have a number of drawbacks including limited availability, requirement of additional surgeries, and the potential for donor site morbidity. Allografts alleviate some of these concerns but can still be limited by donor availability, potential disease transmission, and reduced biological and mechanical efficacy following treatment (*i.e.* terminal sterilization *via* gamma irradiation) (Greenwald et al., 2001). While sterilization effects can vary depending on treatment parameters and bone type, dose-dependent decreases in mechanical properties are likely owed to the degradation of collagen molecules (Nguyen et al., 2007). Various studies have shown decreases in strength (up to 50%) (Godette et al., 1996), work to fracture (up to 70%) (Currey et al., 1997), and fatigue life (up to 87%) (Akkus and Belaney, 2005) of allograft bone following irradiation. As an alternative to grafts, demineralized bone matrix (DBM) has been tested in a number of segmental defect and spinal fusion orthopaedic models with mixed results. DBM is often processed as a powder and delivered to the defect site in gel, putty, or paste form, alone or in combination with autologous bone tissue or biomolecules. This allows for some degree of osteoinduction and bone formation bridging the defect, but does not maintain the complex matrix architecture or provide any early mechanical stability without additional fixation (Bolander and Balian, 1986; Drosos et al., 2007; Iwata et al., 2002; Martin et al., 1999; Pieske et al., 2009; Wang et al., 2007). As a result, there is a need for improved bone replacement constructs to treat non-healing defects (Bueno and Glowacki, 2009; Giannoudis et al., 2005; Logeart-Avramoglou et al., 2005).

This need has motivated the pursuit of improved bone graft substitutes for use in treating bone defects. These constructs are often used at bony sites to bridge tissue defects and support mechanical stresses. Mechanical competence of bone replacement constructs has been difficult to achieve solely using synthetic or natural polymers, and there has been a shift towards composite constructs that combine a rigid mineral phase with a ductile polymer (Wei and Ma, 2004). In an effort to maintain biocompatibility, along with increased mechanical properties and osteoconductivity, numerous studies have incorporated mineral into polymer substrates with promising results (Gu et al., 2011; Liu et al., 2011; Price et al., 2009; Song et al., 2005). Many investigators homogeneously mix mineral powder/crystals with a polymer while others cover porous polymer constructs with a ceramic coating (Al-Munajjed and O'Brien, 2009; Davis et al., 2009; He et al., 2010; Huang and Miao, 2007; Kane and Roeder, 2012; Zhao et al., 2009). The inclusion of mineral and polymer combines the advantages of each material component and this growing trend towards composite materials in bone tissue engineering (BTE) better matches the biphasic composition of natural bone. However, few studies have recapitulated or maintained the natural macrostructure of bone matrix within their tissue engineered constructs.

Bone tissue is a composite material composed of water, an organic matrix, and inorganic mineral components. The organic matrix is primarily composed of type I collagen, a viscoelastic material with low compressive modulus and high tensile strength. The hydroxyapatite (HA) mineral component has relatively high stiffness coupled with low fracture toughness and is brittle when not reinforced by the hydrated organic matrix (Currey, 2002). These two primary constituents have a complex hierarchical structure within bone. Tropocollagen is formed when three collagen molecules assemble into a triple helix. These tropocollagen molecules assemble into a staggered array of fibrils, which ultimately become mineralized as the water within the fibrils is replaced with mineral (Toroian et al., 2007). This intrafibrillar mineralization is critical to the mechanical performance of bone (Kinney et al., 2003).

Demineralized bone matrix (DBM) has high tensile strength (collagen fibers: 0.5-1 GPa in tension (Catanese et al., 1999; Fung, 1993) and natural osteoinductive properties which make it a potentially effective scaffold for bone tissue engineering (Urist, 1965). Integrating a mineral phase could further improve the material by improving both osteoconductivity and compressive strength; however the dense collagen matrix provides a challenge to incorporating mineral. Recently, a number of studies have utilized mineralization inhibitors or other molecules to modulate mineral crystal growth within collagen fibrils. Thula et al. demonstrated a method to remineralize DBM using the “PILP”, or polymer-induced-liquid-precursor method (Thula et al., 2011). By incorporating acidic macromolecules into solution to mimic the non-collagenous proteins (NCPs) naturally present in bone matrix, they were able to achieve a more “biomimetic” mineralization. Nudelman et al., reported intrafibrillar mineralization of 300 nm collagen fibers via the use of a mineralization inhibitor in solution (Nudelman et al., 2010). Recently Price et al. sought to understand the mechanism of intrafibrillar mineralization of type I collagen fibrils and to recapitulate the natural mineralization process using a natural mineralization inhibitor (Price et al., 2009). These studies have achieved successful remineralization of collagen on the nano- or micro-scale, with the goal of mimicking the physiological mineralization process while substituting various biomolecules in place of NCPs. During development, bones start small and mineralize as they grow. Therefore, mineralization inhibitors and other NCPs are able to function because *in vivo* mineralization occurs at a nano-scale, with the hierarchical structure created layer-by-layer by osteoblasts until a macro-sized tissue is ultimately achieved (Jee et al., 2010).

The objective of this study was to develop an approach to efficiently remineralize macro-scale constructs. Initially, we applied the method published by Price et al. to clinically relevant-sized collagen constructs, yet we did not observe mineral deposition in the matrix interior. To address this limitation, we developed a technique for the remineralization of demineralized bone matrix (DBM) based on alternating solution immersion (ASI). This automated technique facilitates the incorporation of mineral into DBM with resultant increases in the mechanical properties greater than 10 fold after 96 hours of treatment. By using a natural, dense, and ordered collagen source such as DBM, we have maintained the inherent tensile strength of the organic phase of bone without destroying the unique architecture of the matrix. Together with the attributes of an inorganic mineral phase (i.e. compressive strength, osteoconductivity), we anticipate this remineralization process will be useful in the preparation of stiff and strong allografts or xenografts for clinical BTE application.

1.2 Methods

1.2.1 Specimen Preparation

Equine third metacarpal (MC3) bones were collected post mortem at the J.D. Wheat Veterinary Orthopedic Research Lab (VORL) at UC Davis. Bones were cut into workable segments (approximately 12 cm long) using a band saw. Rectangular cortical bone beams ($\sim 2 \times 2 \times 22$ mm) were prepared from the diaphyseal portion of MC3 using an Exakt cutting system (Exakt Technologies) and specialized cutting jigs. Cylindrical cores of trabecular bone 6 mm in diameter and more than 10 mm in length were cut from the distal end of the MC3 using a drill press (Model J-2530, JET). Tissue was kept frozen at -20°C when not being machined. The tissue was kept hydrated during machining with deionized (DI) water.

1.2.2 Demineralization

Mineral was removed from MC3 bones via submersion in a demineralizing solution (formic acid (22.5%) – sodium citrate (100 g/L)). This solution has been reported to effectively

remove mineral from bone tissue while preserving the collagen matrix (Yeni et al., 2002). Cortical beam or trabecular core specimens were placed on a shaker in 40 mL of demineralizing solution and the solution was replaced every two days. Beams were contact x-rayed (Model 805, Faxitron Bioptics; 30 kVp for 1 minute) at each solution change to qualitatively assess demineralization. Once demineralization was complete (*i.e.*, specimens no longer appeared white in radiographs), specimens were then placed into fresh demineralizing solution for an extra day. Demineralized specimens were then removed and ammonium oxalate (5% w/v) was added to the solution (1 mL per 5 mL demineralizing solution) to chemically confirm complete demineralization. Following demineralization, remineralization was attempted using two methods.

1.2.3 Remineralization by Inhibitor Exclusion (with Fetuin)

Following decalcification, cortical beams (n=3) were pretreated to enhance remineralization using one of three methods: 1) treatment with 0.5M NaOH; 2) treatment with 0.1% SDS; and 3) no treatment. All beams were rinsed thoroughly for 24 hours with DI water following treatment. Then, cortical beams were remineralized using an inhibitor exclusion method previously described (Price et al., 2009). Briefly, fetuin, a mineralization inhibitor was dissolved (5 mg/mL) in separate solutions of 0.2M HEPES containing 0.02% sodium azide and either 10 mM CaCl₂ or NaH₂PO₄. Solutions were then rapidly mixed together in the presence of NaHCO₃ buffer and the demineralized matrix specimen was added to the solution. The entire calcification solution, with specimen, was placed on a shaker and replaced with fresh solution every 2 days for up to 3 weeks.

1.2.4 Remineralization by Alternating Solution Immersion (ASI)

Demineralized cortical beams were sequentially exposed to .55M calcium chloride solution and 0.5M sodium phosphate solution. Specimens remained in each solution for 35 minutes. Each salt solution was in a separate container on a stir plate. Alternation of solutions was manually performed for 96 hours (n=2). Assessment of x-ray and microCT data from these two remineralized specimens served as pilot data to design the more robust and automated method described below.

1.2.5 Automating Remineralization by Alternating Solution Immersion

Solenoid operated pinch valves (Bio-Chem Fluidics) were purchased to regulate the flow of mineral solutions. An 8-port USB relay control card (EasyDaq Solutions) was used to signal the valves to open and close, and the system was powered by a regulated power supply (HQ Power). The timing of the entire system was controlled with a LabView (National Instruments) program written for this purpose. The automated ASI procedure was performed on separate sets of cortical (n=12) and trabecular (n=5) bone specimens to confirm efficacy in both bone types. Initially, six cortical bone specimens were suspended in a beaker on a heated stir plate. The calcium chloride solution was dispensed into the beaker, allowed to stir for 45 minutes, and then removed via vacuum. The alternate solution (sodium phosphate) was then introduced for 45 minutes and the process repeated under computer control for up to 96 hours. The method was repeated with a second batch of six specimens for a total of twelve remineralized cortical beams. A group of 5 cylindrical trabecular bone specimens was also treated via ASI for 96 hours.

1.2.6 Assessing Remineralization

Cortical beams were imaged throughout the study via contact x-ray. ImageJ (National Institutes of Health) was used, along with a custom Matlab program, to convert the average grayscale value to an apparent radiographic mineral density (in estimated thickness of aluminum (ETA) (Martin et al., 1990) throughout remineralization. MicroCT analysis of

both beams and trabecular cores (55 kVp, 145 μ A, 300 ms integration time, average of 3 images, 6 μ m resolution) was performed to determine the amount (mineral volume fraction (MVF)) and apparent mineral density of the construct using standard SCANCO software (μ CT 35, Scanco Medical). Regions of interest (ROIs) were selected manually for each specimen cross-section as close to the outside edge as possible. A global threshold value of 375 mm HA/cc was used; all pixels above the threshold were considered mineralized and those below were classified as non-mineralized. The image threshold was chosen by examination of the grayscale histograms for each specimen and is consistent with previous studies' imaging of trabecular bone or less dense new bone (Ding et al., 1999; Freeman et al., 2009). Histograms of the grayscale images contained 2 peaks, one at low grayscale values and one at higher grayscale values. We selected a threshold in the valley between these two peaks, thus discriminating between mineralized and non-mineralized tissue (see Supplemental Figure 1). The MVF was defined as the number of voxels above the threshold divided by the total number of voxels in the ROI.

Additionally, both demineralized and ASI-remineralized trabecular specimens were imaged using scanning electron microscopy (SEM; 30 kVp, 60 μ A) to confirm pore structure (see Supplemental Figure 2).

1.2.7 Mechanical Testing

Mechanical characterization of native (nondemineralized), demineralized, and ASI-remineralized constructs was performed using a Bose Enduratec ELF3200 mechanical testing system. Cortical beams were loaded *via* 3 point bending and the stiffness was calculated as the slope of the linear region of the force-displacement curve (see Supplemental Figure 3). Native bone beams were loaded up to 15N, while demineralized and ASI-remineralized beams were loaded to 0.375N. Trabecular cores were loaded between polished steel platens in unconfined compression using a ramp displacement loading. Cores were fixed between two platens and compressed to 1% strain (native) or 10% strain (demineralized and remineralized) at a strain rate of 0.01 sec⁻¹. Stiffness of the specimens was calculated as the slope of the linear region between 25-50% of the maximum load. Bending tests were performed three times on each cortical beam: first on native bone specimens, then following demineralization, and again after 96 hours of ASI-remineralization treatment (n=12). Compression tests were performed twice on each trabecular core: first on demineralized specimens and again following 96 hours of ASI-remineralization treatment (n=5).

1.2.8 Statistical Methods

Repeated measures analysis of variance (ANOVA) was used to compare stiffness values between the cortical groups. Dunn's multiple comparisons test (nonparametric) was used to compare specific differences between groups to account for the non-normal distribution of stiffness measurements of remineralized specimens. Batch number (1,2) was used as a covariate within the ANOVA as half the beams were remineralized in one "batch", half in a second "batch". For trabecular specimens, one-way ANOVA was performed, followed by Tukey's multiple comparisons test for post hoc pairwise analyses. A paired t-test was performed to compare mean stiffness between demineralized and remineralized groups separate from native bone to analyze the effect of treatment. Values are reported as mean $\pm$ standard error of the mean (SEM). All tests were considered significant at $p < 0.05$.

1.3 Results

1.3.1 Remineralization by Inhibitor Exclusion (with Fetuin)

X-ray images revealed a gradual increase in the density of the remineralizing beams when analyzed over the course of three weeks of treatment. However, examination of the mineral distribution showed that mineral deposition was primarily located outside of the boundaries of the demineralized matrix. Qualitative radiography was confirmed using ImageJ to analyze the grayscale values of the radiographs (Fig. 1) and by 3D microCT (Fig. 2). MicroCT data confirm the presence of mineral solely around the edges of the demineralized bone and not throughout the collagen matrix. Mineral was so weakly bound to the specimens that it began to flake off with excessive handling, making mechanical testing of these specimens unfeasible.

1.3.2 Remineralization by Alternating Solution Immersion (ASI)

Two cortical beams were allowed to remineralize using the manual ASI method for 96 hours to test the efficacy of the method. Serial x-rays indicated the accumulation of mineral within the demineralized bone matrix (DBM) over time (Fig. 3A). Following treatment, microCT data confirm that mineral was accumulating within the collagen matrix and not simply coating the outside as in the inhibitor exclusion method (Fig. 3B). Data from these two specimens motivated the design and construction of the automated system, as well as the collection of data from the larger group of specimens described below.

A group of demineralized cortical beams (n=12) was then remineralized via ASI. Remineralized cortical specimens had an average mineral volume fraction (MVF) of 0.381 $\pm$ 0.01. Overall, the beams had an average apparent density of 665.1 $\pm$ 14.46 mg HA/cc for the mineralized regions above the threshold. For comparison, native cortical specimens before treatment had a MVF of 0.928 $\pm$ 0.008 and an apparent density of 1041.7 $\pm$ 13.26 mg HA/cc. Demineralized specimens had a MVF of 0.005 $\pm$ 0.003. MicroCT analysis of remineralized trabecular cores (n=5) (Fig. 4) revealed an average MVF of 0.228 $\pm$ 0.046 compared to the native bone core (n=1) with MVF of 0.55. The average apparent density of the remineralized cores was 670.3 $\pm$ 22.4 mg HA/cc compared to 881.61 mg HA/cc for the native core.

1.3.3 Changes in Stiffness through Remineralization

Three-point bending tests determined the mean stiffness of cortical beams as follows: native = 236 $\pm$ 7.19 N/mm, demineralized = 0.21 $\pm$ 0.01 N/mm, and ASI-remineralized = 2.76 $\pm$ 0.57 N/mm (Fig. 5) (n=12). Remineralized beams exhibited significantly increased stiffness compared to demineralized beams (p<0.05), although both demineralized (p<0.001) and remineralized (p<0.05) groups were less stiff than native bone samples. The differences remained significant following the inclusion of “batch” as a covariate in the analysis. Compression tests determined the average stiffness of the trabecular cores as follows: native = 332.8 $\pm$ 41.4 N/mm, demineralized = 12.1 $\pm$ 2.6 N/mm, and remineralized = 50.7 $\pm$ 13.5 N/mm (Fig. 6) (n=5). ANOVA analysis determined that demineralized and remineralized mean group stiffnesses were significantly different from native trabecular stiffness (p<0.001). However, Tukey’s multiple comparisons test did not find a significant difference between demineralized and remineralized mean group stiffness. A paired t-test comparing the demineralized and remineralized group indicates that mean stiffness of demineralized and remineralized groups are significantly different (p<0.05) when considered separate from the native bone stiffness data, demonstrating an effect of ASI treatment.

1.4 Discussion

In this study we used two methods to remineralize demineralized equine bone matrix. We were unable to demonstrate successful remineralization throughout larger pieces of DBM using a previously reported method based on exclusion by a mineralization inhibitor. However, we successfully demonstrated that an alternating solution immersion method, in which demineralized bone is exposed to alternating, separate calcium ion and phosphate ion rich solutions, causes mineral to form within DBM.

The “mineralization by inhibitor exclusion” method caused mineral formation on the exterior of millimeter sized collagen matrices, rather than within the collagen fibrils and throughout the matrix. It is possible that mineral began to penetrate the collagen matrix on the nanometer length scale as other investigators have reported (Nudelman et al., 2010), however this was insufficient for our goal of macro-scale mineralization. In fact, the mineral layer coating our matrix construct flaked off with handling, further indicating that mineral formation occurred primarily on the surface and not within the bulk of the collagen fibrils. The proposed mechanism for this remineralization method is that fetuin binds to mineral ions to prevent mineral precipitation in the solution. However, fetuin is sterically excluded from the matrix (Toroian and Price, 2008), permitting the calcium and phosphate ions to infiltrate the matrix where they combine and precipitate as mineral. We believe that calcium and phosphate mineral ions are bound to fetuin, but still free to precipitate and form a mineral phase (Heiss et al., 2003). Presumably, this mineral is also able to associate with proteins such as collagen fibrils (Heiss et al., 2008). Since the fetuin-mineral complex is too large to penetrate the collagen fibrils, the mineral begins to nucleate and grow on the surface of the dense collagen matrix, creating a diffusion barrier that limits further crystal growth. This proposed mechanism is consistent with other reports using this technique, which demonstrated mineral penetration of collagen fibrils up to nano- and micro-scale distances, but not throughout the bulk of macroscopic (mm) sized materials with the density and structure of DBM (Nudelman et al., 2010; Thula et al., 2011).

The ASI method addresses this issue by maintaining separate solutions of calcium and phosphate ions, eliminating the need to introduce inhibitory biomolecules, such as fetuin, into the system. The proposed mechanism of this remineralization method is that a single ion type is allowed to diffuse throughout the pores of the DBM, eventually associating with the organic materials within the collagen structure. The solution is then removed, leaving behind ions within the collagen structure (Mann, 2001). The characteristic 64 nm banding pattern of collagen fibrils has been shown to be necessary for CaPO_4 precipitation (Koutsoukos and Nancollas, 1987), and mineral crystals maintain a close spatial relationship with collagen fibrils, specifically the gap zones, during bone mineralization (Hall, 1990). Once the structure is exposed to the alternate solution, the newly introduced ions diffuse into the structure where the natural matrix architecture provides a structural framework and a surface for nucleation of mineral within the construct. This process is repeated many times, allowing the nucleated mineral crystals to grow larger throughout the matrix, creating a composite material of organic matrix and inorganic mineral within the structure. Precise localization of the mineral crystals in the dense matrix has not yet been performed.

The ability to remineralize sections of demineralized bone matrix could be useful as a method of increasing the mechanical properties and osteoconductivity of an implantable construct made from the natural collagenous matrix. Although we demonstrated our method using DBM, we do not foresee a barrier to applying alternating solutions for the mineralization of other protein constructs or to its application to solids. Further, our SEM images demonstrate that pores are still present in the trabecular matrices following 96 hours

of ASI-remineralization (Supplementary Figure 2). This indicates that cell seeding and infiltration could be possible in future studies.

This project serves as a proof of concept for ASI, confirming that it can form mineral within a large, dense collagen construct such as DBM. In this study, we demonstrated an ability to incorporate mineral throughout equine DBM using ASI. Qualitatively, DBM is rubbery and highly flexible while our remineralized construct is palpably more rigid. The flexibility prior to remineralization suggests this method will provide new opportunities to prepare shape-specific mineralized constructs. Quantitatively, ASI increased the stiffness of DBM cortical beams approximately 13 fold under the current conditions. This measured stiffness remains well below our measured stiffness of native bone, but we believe the significant increase in stiffness will improve the ability to handle this material in future bone graft applications. A number of studies on the mechanical properties of either collagen or synthetic polymer/hydroxyapatite composite scaffolds have reported comparable and slightly lower mechanical properties for both compression and bending tests, indicating that not only does the ASI process significantly improve mechanical properties of DBM, but it allows for fabrication of constructs with modestly improved properties compared to materials recently reported in the literature (Al-Munajjed and O'Brien, 2009; Bakos et al., 1999; Huang and Miao, 2007; Kane and Roeder, 2012; Kikuchi et al., 2004; Yunoki et al., 2011). There was a large variation in the reported stiffness data of our ASI remineralized specimens, indicating the heterogeneous nature of the remineralization procedure. It is known that bone contains additional components such as cells, lipids, and non-collagenous proteins (NCPs). We anticipate that our demineralizing procedure removes some of these materials from the matrix along with the mineral; however any remaining molecules (i.e. NCPs, mineralization inhibitors, lipids) could affect the remineralization results. We are currently exploring techniques to identify and remove these additional materials prior to remineralization of the DBM in order to improve the uniformity of mineralization.

In this study, a natural bone collagen matrix provided the framework for the nucleation and growth of the mineral phase. Our goal was to grow a mineral phase within the volume of native tissue matrix. By using a dense and highly organized collagen source such as DBM, we maintained the natural tensile strength of the organic phase of bone without destroying the unique architecture of the matrix. While other groups have shown the ability to coat synthetic matrices with mineral or incorporate mineral onto relatively thin collagen/DBM constructs (Gu et al., 2011; Liu et al., 2011; Liu et al., 2011; Price et al., 2009; Song et al., 2005; Thula et al., 2011), we have demonstrated a technique for incorporating a mineral phase within thick (up to 2mm) natural cortical and trabecular (6 mm) bone matrices. Our technique is unique in its simplicity and its automation. We believe that our ability to create a composite material with the appealing attributes of native bone matrix (i.e. high tensile strength, osteoinductivity) and the inorganic mineral phase (i.e. compressive strength, osteoconductivity) demonstrates the potential of this technique for use in the manufacturing of clinically useful materials for bone repair. Further, the automated nature of this process facilitates user control of various system inputs including the addition of complementary solutions of proteins or growth factors that could improve the utility of the final product.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

BTE	bone tissue engineering
DBM	demineralized bone matrix
ASI	alternating solution immersion
RBM	remineralized bone matrix
MC3	third metacarpal bone
HA	hydroxyapatite
MVF	mineral volume fraction
SDS	sodium dodecyl sulfate

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- Scaffolds often lack desired mechanical properties for bone tissue engineering
- We examine methods for incorporating mineral throughout demineralized bone matrix
- Alternating solution immersion (ASI) is a novel method to remineralize bone matrix
- Mineral inclusion increases demineralized matrix stiffness by an order of magnitude
- Remineralized bone matrix has potential for use in graft applications

FIGURE 1.

A. Representative x-ray images of demineralized and remineralized bone matrix prepared using the inhibitor exclusion method. Top image of each panel depicts an initial x-ray of a fully demineralized matrix. Bottom image of each panel depicts an x-ray following up to three weeks of the “mineralization by inhibitor exclusion” treatment. Mineral shows up as a lighter color (higher grayscale value). B. Representative x-ray image of partially remineralized bone matrix, analyzed for mineralization profile using ImageJ. The white line in the top image was selected manually and indicates region of analysis. Spikes in grayscale in bottom image indicate higher mineral content on the edges of the matrix.

FIGURE 2.

3D representative microCT images of DBM following three weeks of treatment using the “mineralization by inhibitor exclusion” method, along with native (untreated) bone. Top row: Cross-sectional view, looking at end of construct. Bottom row: Full view of construct. Note the shell-like appearance and lack of mineral present within the bulk of the collagen matrix for all treatment groups. Scale bar applies to all 8 panels.

A

B

FIGURE 3.

A. Serial x-ray images of 2 bone beams and an aluminum step wedge standard. Initial x-ray (of demineralized beams) on the left, final x-ray (after ASI-remineralization for 96 hours) on right. B. MicroCT images of a single ASI-remineralized bone beam following 96 hour treatment. Left: Cross-section through short axis; center: Cross-section through long axis; right: 3D visualization of mineral present in the beam.

FIGURE 4.

Remineralized trabecular cores. Left column: representative transverse slices through 2 remineralized cores (top 2 panels) or one native trabecular core (bottom panel); Right column: 3D visualization of mineral present within trabecular core (top panel = longitudinal slice through remineralized specimen, middle panel = transverse slice through remineralized specimen, bottom panel = gross view of native specimen).

FIGURE 5.

Average stiffness values of 12 cortical beams measured in 3 point bending before and after ASI-remineralization (error bars represent $\pm$ SEM). Stiffness is significantly different between groups (* $p < 0.05$; ** $p < 0.001$). Note: mean stiffness shown on y-axis in log scale.

FIGURE 6.

Average stiffness as measured in compression for native, demineralized, and remineralized trabecular specimens (error bars represent $\pm$ SEM). Demineralized and remineralized mean group stiffnesses were significantly different from native trabecular stiffness (** $p < 0.001$). Remineralization treatment significantly increases mean stiffness (* $p < 0.05$). Note: mean stiffness shown on y-axis in log scale.