UNIVERSITY OF CALIFORNIA, SAN DIEGO

A New, Potential Mechanism for Osteoblast Initiated Lamellar Bone Formation During Bone Remodelling

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in

Biology

by

John Se Kit Yuen Jr

Committee in Charge:

Professor Paul Price, Chair
Professor James Kadonaga
Professor Milton Saier

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The Thesis of John Se Kit Yuen Jr is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

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EPIGRAPH

"Only those who dare to fail greatly can ever achieve greatly."

Robert F. Kennedy

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ABSTRACT OF THE THESIS

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by

John Se Kit Yuen Jr

Master of Science in Biology

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Professor Paul Price, Chair

Throughout one's life a person's bone constantly goes through bone resorption and bone formation in a process called bone remodelling. Bone diseases such as osteoporosis are caused by a dysregulation of this bone remodelling. Bone resorption is well understood, but the molecular mechanism and biology of bone formation is not fully understood. It is known that osteoblasts (bone forming cells) form a 5-10 um layer of osteoid (bone matrix) and calcify (mineralize) it to form bone. However, the exact process by which osteoblasts calcify osteoid is not fully understood. In this study we show that it is possible for lamellar bone formation to occur via a two step "seed-grow"

mechanism. In this mechanism osteoblasts first seed osteoid with many small mineral crystals, after which the seed crystals go on to grow and calcify osteoid to form the final bone tissue. The seed crystals grow by taking calcium and phosphate from the circulating blood that bathes osteoid and therefore do not require osteoblasts for mineral growth past the initial seeding step. The seed-grow mechanism was tested via a trabecular bone block model system, where blocks of bovine trabecular bone were first decalcified (demineralized) to recreate the initial osteoid material (bone collagen blocks). Bone collagen blocks were then artificially seeded with many small crystals via a previously developed technique and incubated in adult bovine serum without osteoblasts. The bone collagen blocks recalcified (remineralized) and hardened, eventually forming a complete, freestanding mineral continuum – a characteristic of the mineral phase in normal bone.

INTRODUCTION

Bone is a multipurpose composite that is – by weight – 20% bone collagen (type I collagen), 70% bone mineral (carbonated hydroxyapatite), 10% water and other constituents [1]. Hydroxyapatite mineral has a high compressive strength [2], allowing bone to bear both the weight of our bodies and the various loads that we carry. Type I collagen has high tensile and yield strength [3], allowing bone to resist being pulled apart and giving bone the ability to bend without permanent deformation. The two bone constituents are organized together at the nanometer scale, truly blending each material's structural characteristics and creating a versatile composite that can handle a variety of stresses (Figure S1).

In the body, bone is constantly being remodelled to maintain its structural integrity and to regulate blood calcium levels. This lifelong process occurs as bone cells remove (resorb) and reform portions of existing bone, fixing microfractures and modulating the balance between resorption and formation to maintain blood calcium homeostasis [4]. A disruption of the balance between bone resorption and formation leads to bone diseases such as osteoporosis, where bone loss occurs due to too much bone resorption, too little bone formation or both [5]. Since a disruption of either resorption or formation can lead to bone disease, effective treatment necessitates an in depth understanding of both processes. Much is known about bone resorption [6]. However, the precise details of how bone formation takes place are not well understood [4].

In Figure 1A we illustrate what is known about the process bone formation. Bone formation that occurs during bone remodelling begins when a sheet of bone forming cells called osteoblasts first lay down and crosslink a roughly 5-10 μm layer of type I collagen,

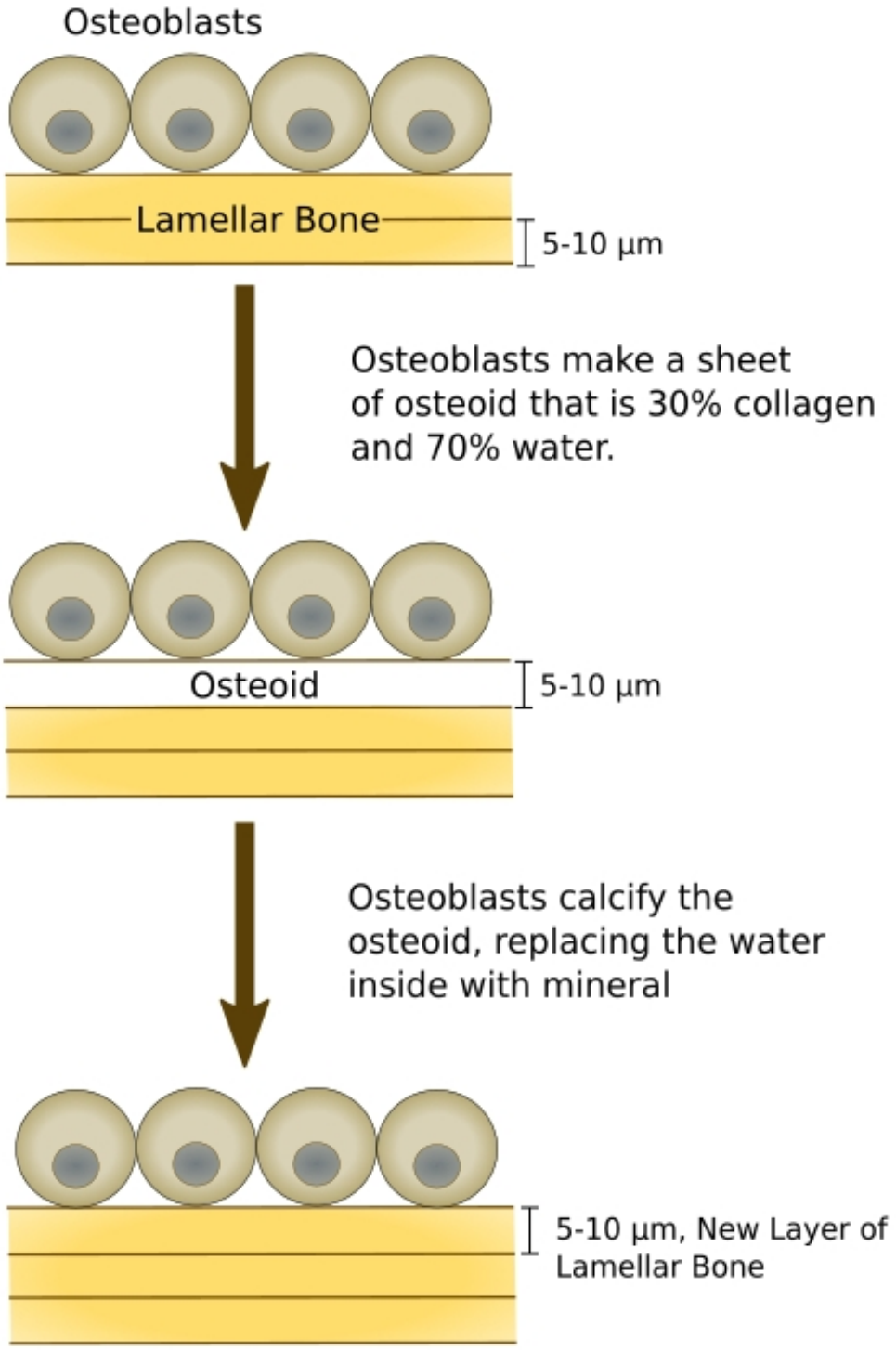


Figure 1A. A Cycle of Lamellar Bone Formation by Osteoblasts. Osteoblasts repeat this cycle of osteoid formation and calcification to form each layer of lamellar bone, the dominant type of bone in vertebrates.

the principal organic component of bone [7][8][9]. This layer is called osteoid (bone matrix) and is observable under light microscopy; it is predominantly type I collagen and roughly 62% water by weight [10][11]. After producing the 5-10 μm layer of osteoid, osteoblasts stop further production of osteoid and begin calcifying (also referred to as mineralizing) its collagen to replace the water and form the final bone tissue. As osteoid is calcified via the displacement of water already present within the collagen, calcification does not alter the shape of the final bone tissue [12]. The completed layer of bone tissue retains its 5-10 μm thickness. This constitutes one cycle of bone formation and is repeated to create successive layers of bone tissue, resulting in an appearance similar to that of rings on a tree [13]. With roughly 10% of an individual's bones being remodelled every year, adult bone largely exists as bone formed through this layer-by-layer process [14]. This is also why mature bone is given the name lamellar bone.

While it is known that osteoblasts lay down osteoid and subsequently calcify it, the exact molecular mechanism by which osteoblasts calcify osteoid is unknown. In this study we present a hypothetical mechanism by which this could take place (Figure 1B). Our proposed mechanism – termed "seed-grow" - involves the calcification of osteoid via a two step mechanism. During the first step, osteoblasts introduce many small mineral crystals into osteoid's collagen matrix. These crystals act as "seeds", serving as starting points for the subsequent mineral growth that fully calcifies osteoid into bone. During the second step, the mineral seeds use calcium and phosphate (constituents of bone mineral) present in circulating blood to grow larger. The mineral crystals eventually fuse with one another and fill the osteoid with mineral. This forms a solid mineral continuum (see Figure 2) and the final bone tissue. Osteoblasts are not necessary for mineral growth

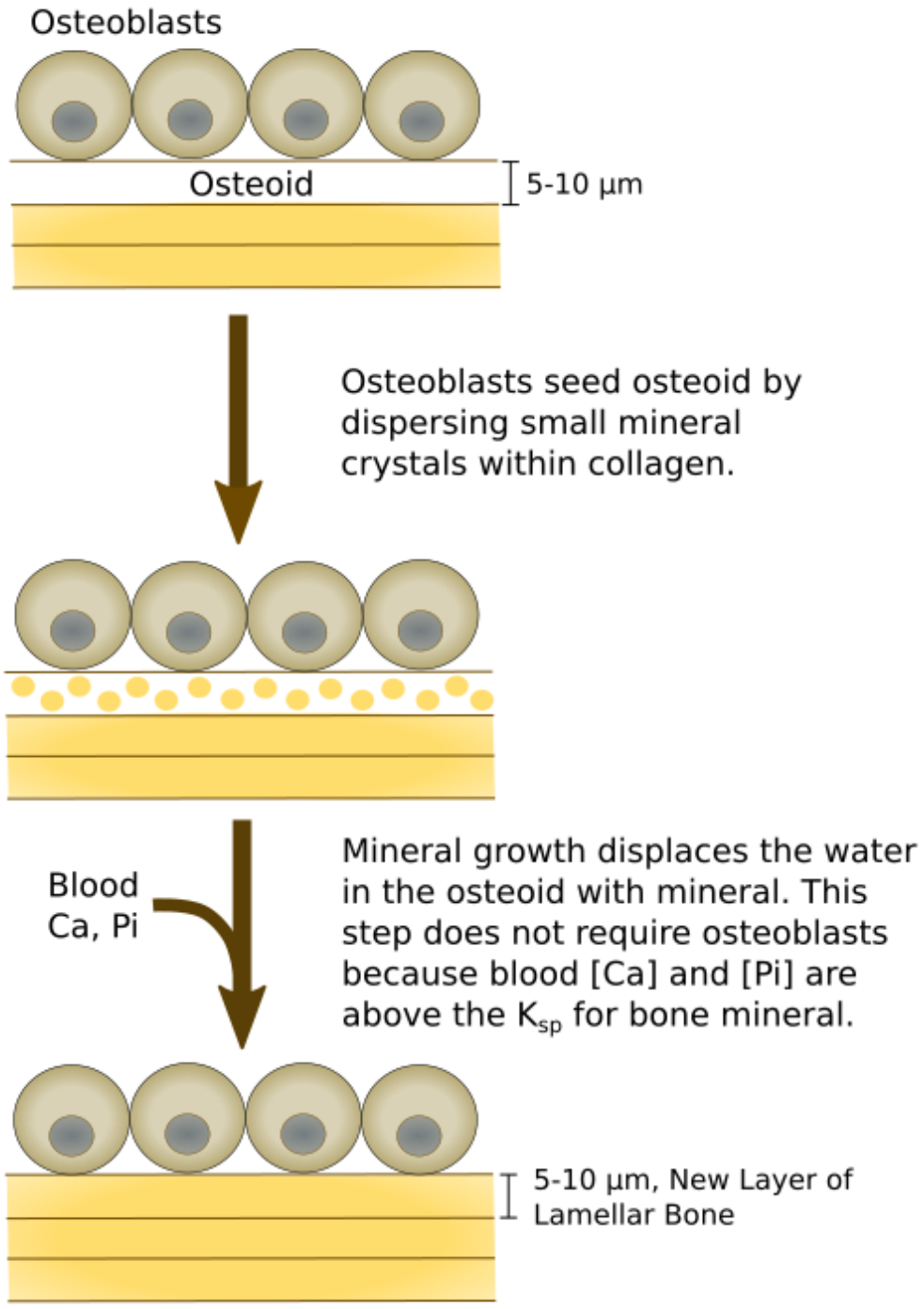


Figure 1B. Proposed Mechanism for Osteoid Calcification. We propose a two step "seed-grow" mechanism for osteoid calcification; osteoblasts first seed osteoid with many small mineral crystals. Crystals then grow and fuse with one another, filling the osteoid with mineral to form the final bone tissue. This process is driven by blood calcium and phosphate and does not require osteoblasts.

during this stage but may perform other tasks that influence calcification [15][16]. Osteoblasts are not necessary for continued mineral growth because osteoid is constantly in contact with blood that circulates in bone, allowing it to be constantly bathed in a solution with calcium and phosphate concentrations that are above the solubility product for bone mineral. The initial seeding by osteoblasts is still necessary, though, because blood calcium and phosphate are only high enough to grow existing mineral crystals, not to spontaneously form new crystals [17]. This is analogous to crystals grown for x-ray crystallography, where a few crystals are nucleated and then grown to size in a solution that is concentrated enough to grow the nucleated crystals, but not concentrated enough to spontaneously create more crystals [18].

As osteoid is not readily available for use in experiments, in this study we test the seed-grow method of osteoid calcification via a trabecular bone block model system. In this system we begin with blocks of trabecular bone sawed from the proximal femoral head of an adult steer and decalcified in 0.15M hydrochloric acid (HCl) to remove all bone mineral (Figure 2). This creates blocks of flexible, translucent, trabecular bone-shaped bone collagen that represents osteoid and serves as the starting material in our seed-grow experiments. Bone collagen is the material of choice because bone is calcified osteoid; decalcification returns the osteoid to its original state. Moreover, the shape of the narrow trabeculae in the decalcified bone collagen block (less than 1 mm wide) provides a large surface area with good diffusional access for calcium and phosphate to enter and form bone mineral. The initial process of decalcifying intact bone to achieve our starting material also allows us to determine the amount of mineral originally present in original bone sample. This allows us to compare the extent of bone recalcification achieved in our

experiments with the extent of calcification in the original bone sample.

In addition to decalcifying trabecular bone blocks, bone blocks can be deproteinized to remove the collagen phase and observe the mineral phase of bone. Figure 2 depicts the deproteinization of initial, untreated bone, showing a solid mineral continuum that maintains its structure even after the removal of bone's collagen matrix. Recalcified bone blocks can also be deproteinized to investigate the mineral phase and see whether recalcification via a seed-grow mechanism recreates bone's sold mineral continuum.

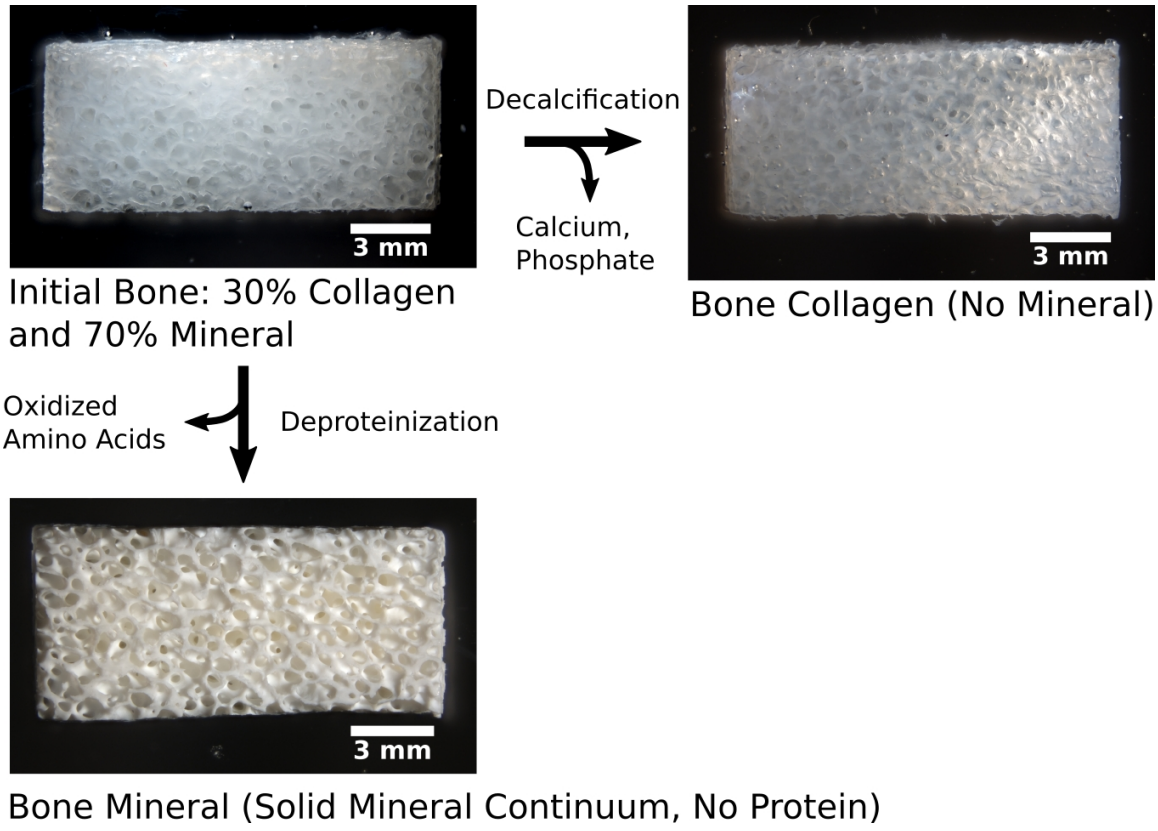


Figure 2. Bone and mineral form independent structures. Bone from the proximal femoral head of an adult steer was freed of marrow and sawed into 5x6x14 mm blocks to create the initial bone used in subsequent experiments. Deproteinization (Removal of collagen) is achieved via immersion in 4% NaOCl, while decalcification is achieved via mixing in 0.15M HCl. Each image is a unique block of bone selected to represent the different procedures.

MATERIALS AND METHODS

Bone Acquisition and Processing

The proximal femur of an adult steer was sawed into 14 mm slabs by a local butcher. Each slab was then sawed into 6x14 mm strips with a hacksaw and a jig. The bone marrow was then removed from the strips with hot water and a dental water jet (Waterpik Ultra Water). After bone marrow removal the strips were hacksawed with a jig into blocks of 5x6x14 mm. The blocks were cleaned with the dental water jet once more to remove debris from the sawing process. After processing, bone blocks were stored in 100 mM sodium phosphate pH 7.4.

Decalcification

Each block of trabecular bone was weighed and decalcified overnight in a 50 ml polypropylene conical tube with 50 ml of 0.15M HCl on a rotator (End-over-end mixing). After decalcification blocks were washed and stored in H_2O. Calcium concentrations of the HCl used during decalcification were measured to determine the amount of calcium originally present in the bone.

Determination of Calcium Concentration

The calcium concentrations of different solutions were determined colorimetrically with a cresolphthalein complexone assay [19][20]. In the assay 4 µl of sample was added to 200 µl of assay reagent and measured for absorbance at 575 nm with a spectrophotometer. The absorbance was compared to a set of calcium standards, also measured at 575 nm. The assay reagent was a 1:1 mixture of a 9.6 vol% 2-amino-2-methyl-1-propanol (Fluka Analytical), 2 mM KCN buffer reagent and a 0.24M HCl, 16.9 mM 8-hydroxyquinoline (Sigma Aldrich), 55 µM o-cresolphthalein complexone (Fluka

Analytical) color reagent.

Seeding

Decalcified bone collagen blocks were seeded to introduce small mineral crystals into the collagen matrix, as described in [21]. In detail, bone collagen blocks were added to 1 to 6 ml pH 7.4 MOPS buffer with 5 mM calcium chloride ($CaCl_2$), 5 mM sodium phosphate (Na_2HPO_4), 30 mM sodium bicarbonate ($NaHCO_3$), 0.02% sodium azide (N_3) and 50 mg/ml bovine fetuin (Sigma Aldrich) in a 10x75 mm test tube. Test tubes were placed on a rotator at room temperature overnight. Seeded bone collagen blocks were washed and stored in calcium and magnesium free Dulbecco's phosphate-buffered saline (DPBS, Gibco Life Technologies) before further use. Amount of calcification achieved through seeded was determined via measuring the change in the seeding solution's calcium concentration before and after seeding. Bone collagen blocks were seeded from 0.3 to 4% of the amount of calcium initially the bone sample.

Staining for Calcium Phosphate Mineral

Bone blocks were mixed in 10 ml of 0.5% potassium hydroxide (KOH) and 0.002% Alizarin Red S (GE Healthcare) overnight in a 15 ml polypropylene conical tube to stain for calcium phosphate mineral. Tubes were placed on a rotator during the overnight staining. After staining blocks were placed in 10 ml of 0.05% KOH to rinse off extra alizarin red stain. Bone blocks were stored in DPBS after staining, regardless of whether the blocks took up stain.

Recalcification

Seeded and unseeded bone collagen blocks were added to 50 ml of adult bovine serum (ABS, Gibco Life Technologies) in a 50 ml polypropylene conical tube and

attached horizontally on a rotator at 37ºC with rubber bands to reduce bubble formation. Tubes were mixed on the rotator for 1-2 days for each cycle of recalcification with ABS. ABS was first prepared by an addition of 1 ml 10% N_3 and 5 ml penicillin streptomycin (Gibco Life Technologies) to 500 ml of ABS. ABS was then aliquotted into 50 ml volumes and frozen at -20ºC. The day before use, a 50 ml volume of ABS were thawed at 37ºC and equilibrated overnight at 5% CO_2 and 37ºC to stabilize pH. After equilibration, ABS tubes were cooled on ice for 10 minutes prior to use. Extent of calcification during each cycle was calculated by measuring the change in calcium concentration before and after each calcification cycle. On the other hand, extent of calcification for unseeded bone collagen was tested by staining for mineral with alizarin red at the end of all calcification cycles.

Deproteinization

Each block of trabecular bone was placed in its own well in a 6 well plate. 10 ml of 8.25% NaOCl (Clorox) was added and left for 6 hours until bubbles stopped coming out of the bone block. A second 10 ml of 8.25% NaOCl was then added and left for 12 hours. After deproteinization, each bone block was washed with two volumes of 6 ml ethanol. The remaining bone mineral was left to air dry after removal of the second ethanol wash.

Imaging

Bone or bone collagen blocks were placed in 6 well plates, submerged in DPBS or H_2O, and photographed on a stereoscope (Carl Zeiss Axio Zoom.V16). Deproteinized blocks were photographed while dry. 4% remineralized bone was deproteinized and stored in ethanol for scanning electron microscopy (SEM, Carl Zeiss Sigma 500).

Hardness assessment

Hardness of bone collagen, recalcified bone collagen and initial, untreated bone blocks was assessed by a single handler. Each bone was lightly squeezed between two fingers and ranked from 1 to 10 in terms of hardness. A score of 1 represented pure bone collagen, while a score of 10 represented initial, untreated bone blocks.

RESULTS

Experimental outline to test the seed-grow hypothesis

Figure 3 shows the experimental approach we used to test if calcification of osteoid is possible via a two step, seed-grow mechanism. As there is no readily available source of osteoid, we have used decalcified bone collagen blocks as a model. Trabecular bone blocks are first decalcified into bone collagen blocks to recreate the osteoid that osteoblasts hypothetically seed with small mineral crystals (See Figure 2). Seeding (Introduction of small mineral crystals) of the bone collagen blocks is then simulated via a previously developed collagen calcification technique termed "Mineral by Inhibitor Exclusion (MIE)" [21]. The technique involves mixing collagen in a seeding solution containing fetuin A (An inhibitor of mineral crystal formation) with calcium and phosphate at levels supersaturated enough to induce a formation of mineral crystals. Fetuin A is too large to enter collagen fibrils, forcing spontaneous mineral formation to only occur in the interior of the collagen matrix [22].

After seeding, bone ollagen blocks are incubated with adult bovine serum (No osteoblasts) to test whether seeded bone collagen is able to passively continue calcification under the physiological conditions that osteoid is usually exposed to in the body (Where calcium and phosphate levels are above the solubility product of bone mineral). Presence of recalcification will be determined by the measured uptake of calcium by the bone collagen blocks, the reformation of a solid mineral continuum, as well as the rehardening of the bone block structure

Seeding bone collagen distributes mineral uniformly throughout the matrix

The quality and uniformity of bone collagen seeding via MIE was assessed by

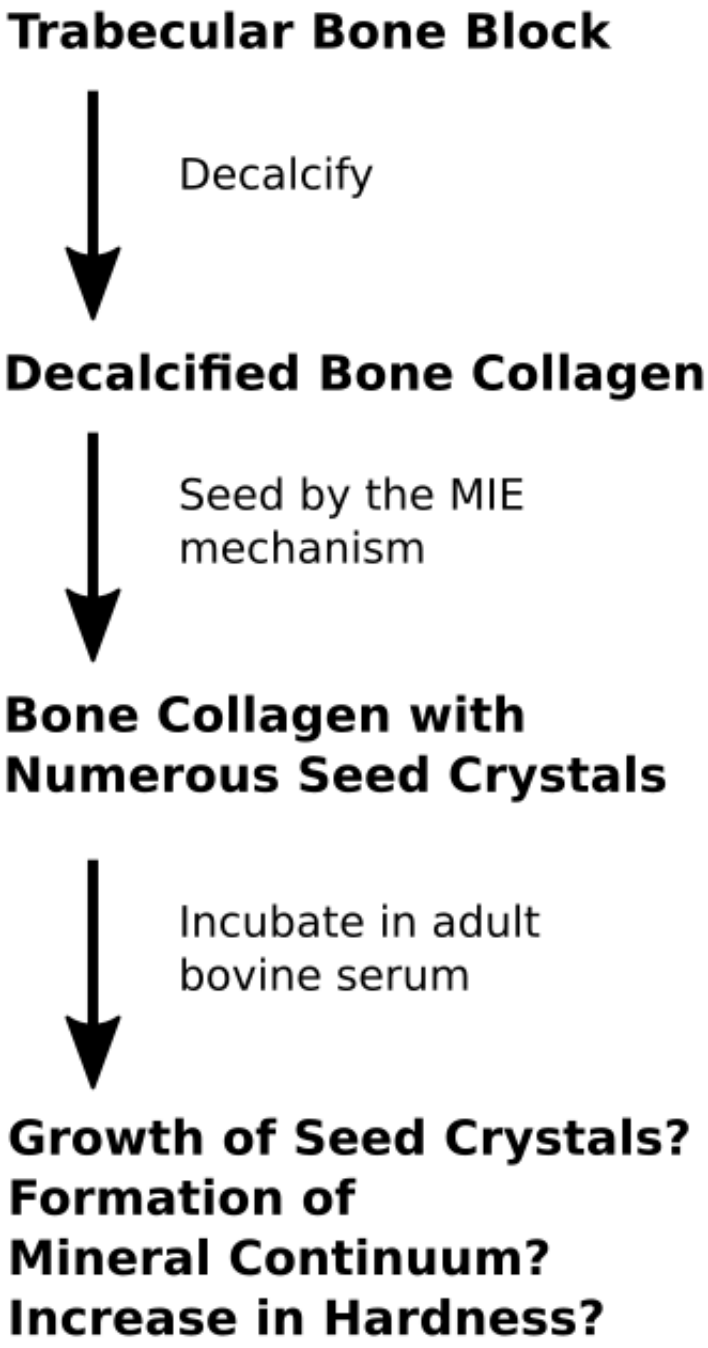


Figure 3. Experimental test of the seed/grow hypothesis.

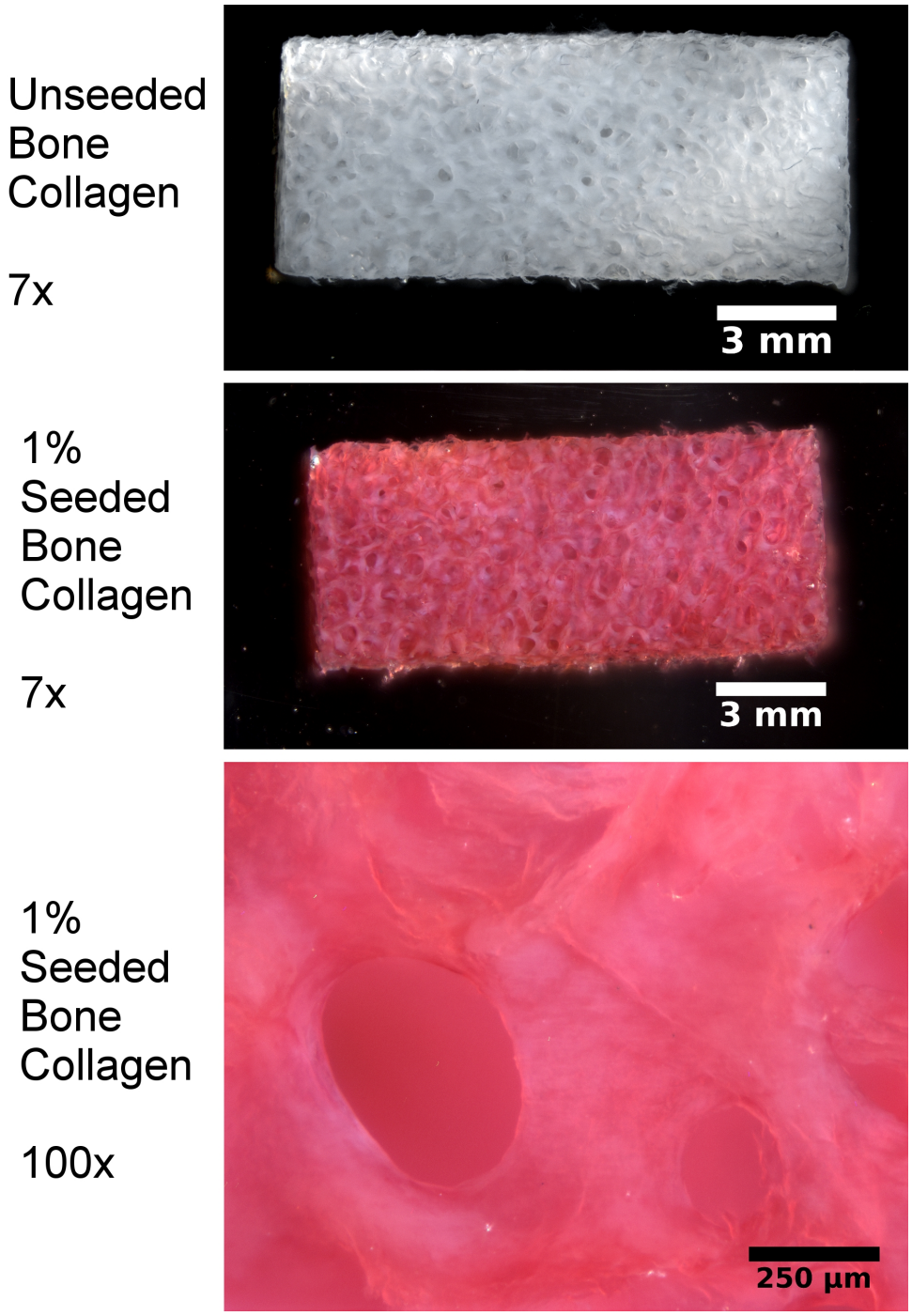


Figure 4. Alizarin Red staining demonstrates that mineral seeds are uniformly distributed throughout bone collagen. Bone collagen was "seeded" to roughly 1% of its original calcium content. Seeding was performed via mixing in a pH 7.4 buffer containing enough calcium and phosphate to spontaneously form mineral crystals. Seeded bone collagen was subsequently stained with alizarin red to investigate the seeding procedure's ability to evenly distribute mineral crystals in collagen.

staining for calcium phosphate mineral using alizarin red. A bone collagen block seeded to ~1% of its original calcium content (1% seeded bone collagen) and an unseeded bone collagen block were stained with alizarin red (Figure 4).

Seeded bone collagen was uniformly stained red, while unseeded bone collagen did not take up any red stain. The uniformity of staining was further confirmed by imaging at a higher magnification, where seeded bone collagen retains a fairly even distribution of red stain. An even alizarin red staining shows that mineral has been uniformly distributed throughout the seeded bone collagen matrix.

Only seeded bone collagen recalcifies in osteoblast-free adult bovine serum

Seeded (1% of initial calcium) and unseeded bone collagen blocks were mixed in 50 ml of adult bovine serum without osteoblasts for 10 calcification cycles (Figure 5). Each cycle involved a fresh 50 ml of adult bovine serum and 1 to 2 days of incubation at 37^{o}C on a rotator. Measurements of calcium concentration in adult bovine serum before and after each calcification cycle were used to calculate the amount of calcium taken up by seeded bone collagen during each cycle. Adult bovine serum mixed at 37^{o}C without seeded bone collagen for 1 to 2 days does not experience a decrease in calcium levels (Data not shown). A total of 4 replicates were performed for seeded bone collagen – one example of the four is shown in Figure 5. Unseeded bone was simply incubated for 10 cycles and stained for alizarin to check for the presence of mineral growth.

Seeded bone collagen recalcified fairly linearly with each calcification cycle, reducing the calcium concentration of each cycle's adult bovine serum by a fairly constant amount (Data not shown). No visible precipitation of mineral was ever observed

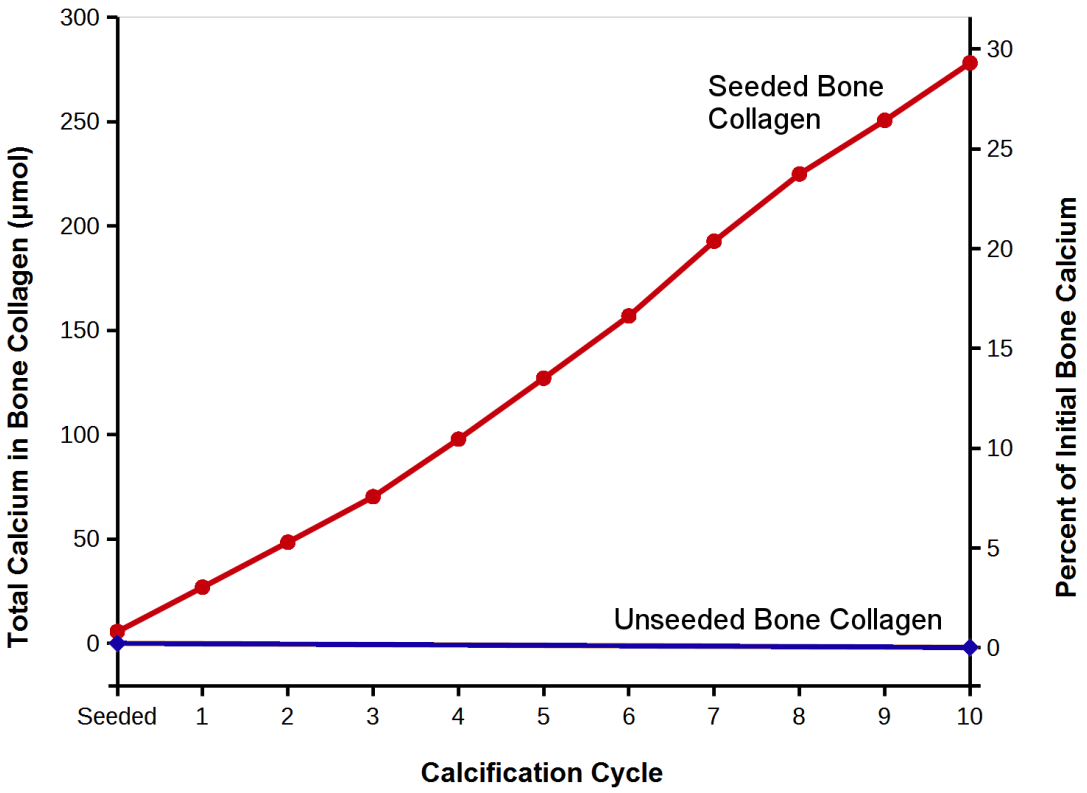


Figure 5. Seeding with crystals is necessary for recalcification in adult bovine serum. Seeded (1% of initial calcium) and unseeded blocks of bone collagen were incubated with mixing at 37°C in 50 ml of adult bovine serum. A cycle of incubation lasted 1-2 days and a total of 10 cycles were performed. The level of calcium uptake per cycle for seeded bone collagen was measured via the drop of [Ca] in adult bovine serum before and after each calcification cycle. One example from four replicates is shown here. Unseeded bone collagen was incubated for 10 calcification cycles and stained with alizarin red to check for mineral growth.

in the adult bovine sera at the end of each cycle. After 10 calcification cycles, seeded bone collagen was calculated to have regained 29.3% of its initial bone calcium. On the other hand, unseeded bone collagen did not recalcify at all, failing to take up any alizarin red stain after 10 cycles of calcification. Only seeded bone collagen appears to recalcify when incubated in adult bovine serum.

Recalcification eventually restores bone's solid mineral continuum

Roughly 1% seeded bone collagen blocks recalcified in adult bovine serum to 13% and 40% of initial bone calcium were deproteinized in two 10 ml volumes of 8% NaOCl and photographed (Figure 6). At 40% recalcification bone's solid mineral continuum appears to have been largely reformed, with only small mineral fragments breaking off of the fused main structure. The fused structure already looks like that of deproteinized initial bone, both retaining the sponge like shape of trabecular bone. Meanwhile, at 13% recalcification a complete mineral continuum is absent, with the mineral phase falling apart into pieces during deproteinization. Deproteinization of seeded bone collagen before recalcification yields a fine white powder that is difficult to see (Data not shown).

Figure 7 shows depicts the 13% and 40% recalcified and deproteinized blocks in Figure 6 at a higher magnification. The bone mineral in 13% recalcified bone appears to be at an earlier stage of mineral fusion, forming shapes that resemble the fused mineral structure of initial bone and 40% recalcified bone, except thinner and broken apart. The 8% NaOCl deproteinization procedure was separately tested for its ability to completely deproteinize bone blocks. An untreated, intact bone block was deproteinized with the same procedure and demineralized in 0.15M HCl to remove the mineral phase

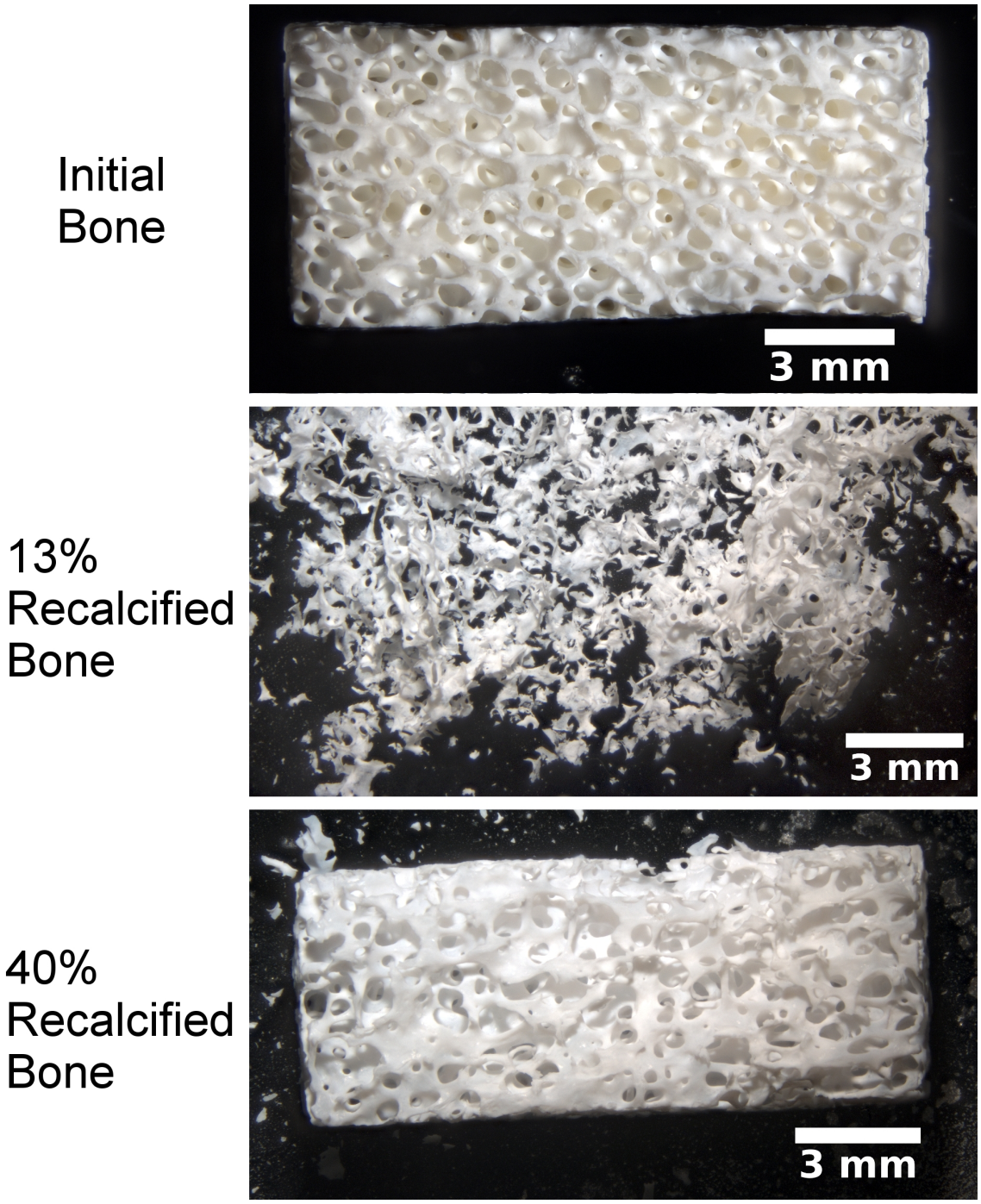


Figure 6. Recalcification of bone in adult bovine serum reforms its solid mineral continuum. An untreated bone block and seeded bone collagen blocks recalcified to 13% or 40% of their original calcium content were each deproteinized in two 10 ml volumes of 8% NaOCl to remove the collagen matrix and leave only the bone mineral. The remnant mineral in 40% recalcified bone shows an almost complete mineral continuum, while the remnant mineral in 13% recalcified bone has collapsed into small pieces.

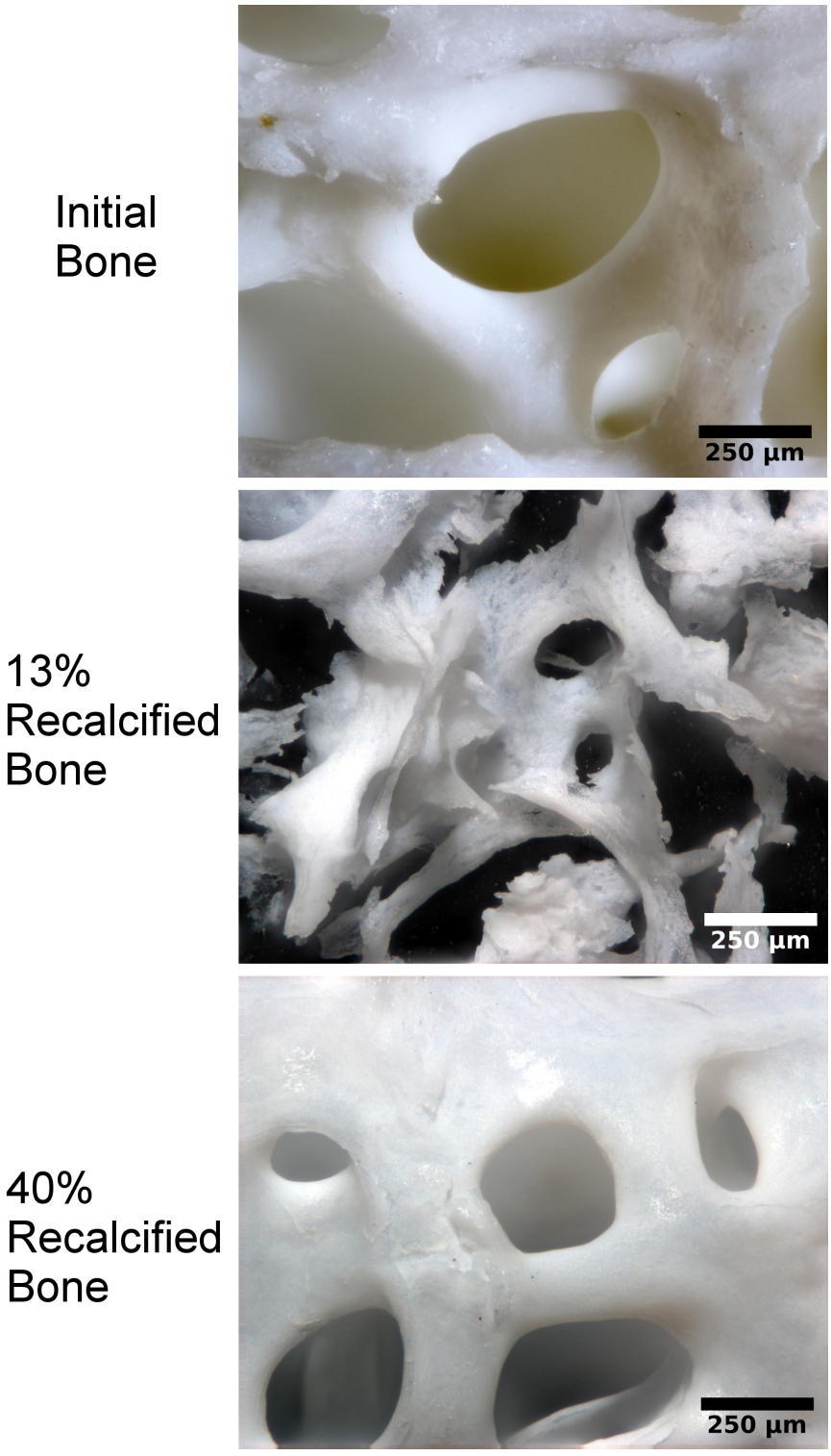


Figure 7. 13% recalcified bone is in the beginning stages of forming a solid mineral continuum. The initial bone block and recalcified-deproteinized blocks shown in Figure 6, but with a specific portion photographed at a higher magnification.

and check for any protein remnants. No protein remnants were seen (Data not shown).

Bone collagen hardens as it becomes more recalcified

The hardness of bone collagen, intact bone and bone collagen calcified to various degrees (1 to 40%) were assessed and graded on a 10 point scale. 1 point represented the softness of unseeded bone collagen, while 10 points represented the hardness of the untreated, initial bone. Table 1 shows the progression of hardness as seeded bone collagen becomes more and more calcified. Seeding bone collagen to 1% calcification does not appear to alter its hardness, leaving a soft and flexible material. Subsequent recalcification however, does appear to result in a rapid increase of hardness. Calcification of seeded bone collagen from by 12% (From 1 to 13%) increased hardness by 33% (From 1 to 4 points). This rapid increase does appear to slow down though: It took another 27% increase in calcification (From 13% to 40%) for hardness to increase by another 33% (From 4 to 7 points). Interestingly, most of bone's initial hardness (7 out of 10 points) seems to be regained by 40% recalcification.

Table 1. Bone collagen becomes progressively harder as the amount of mineral increases. The hardness of untreated (100% mineral) and recalcified (0-40% mineral) bones were assessed by lightly squeezing the samples between two fingers. Seeding bone collagen (1% mineral) does not change its hardness, while recalcifying to 40% mineral appears to give bone most of its original hardness.

Sample	% Calcified	Hardness (1-10)
Bone Collagen Only	0	1
Seeded Collagen	1	1
Seeded Collagen + Serum	13	4
Seeded Collagen + Serum	40	7
Initial Bone	100	10

DISCUSSION

This study aimed to test a two step "seed-grow" mechanism by which osteoblasts might calcify osteoid in the body to form bone tissue. The first "seed" step was achieved via a previously developed technique to disperse small mineral crystals into collagen termed "Mineral by Inhibitor Exclusion" (MIE). The introduction of mineral seed crystals by MIE represents a process that we propose osteoblasts perform during osteoid calcification and bone formation. MIE was tested to ensure that seed crystals were uniformly introduced into bone collagen and that subsequent mineral growth could occur without issues (Figure 4). The second, passive, "grow" step (Not requiring osteoblasts) was achieved by exposing seeded bone collagen to physiological conditions via mixing in osteoblast-free adult bovine serum. Based on the successful calcification of seeded bone collagen that is incubated under physiological conditions, we suggest that osteoblast seeding followed by osteoblast-free growth is a possible mechanism for osteoid calcification and bone formation in the body.

Mineral seed crystals in seeded bone collagen continue to grow and calcify collagen under physiological conditions

Growth of mineral seed crystals (calcification) after incubation in osteoblast-free adult bovine serum (ABS) was confirmed by monitoring the uptake of calcium by seeded bone collagen blocks, the formation of a solid mineral continuum and an increase in hardness. Calcium content in seeded bone collagen increased linearly as each cycle of calcification in ABS passed, with no signs of slowing down after 10 cycles and after regaining 29.3% of initial bone calcium (Figure 5). This suggests that further calcification is possible, perhaps even to the point where all bone mineral is restored. A potential

source of uncertainty in determining the "% of initial bone calcium" in seeded bone collagen is its calculation via the drop in ABS calcium concentration before and after each calcification cycle. We infer the uptake of calcium by seeded bone collagen via the drop of calcium in ABS. Ultimately however, the numbers obtained from the drop in ABS calcium levels are comparable to numbers obtained when recalcified objects have all their mineral removed (via decalcification) and directly measured [23].

The eventual formation of a solid mineral continuum supports the hypothesis of mineral growth taking place on different points (at each seed crystal) in the collagen matrix, eventually growing large enough to meet and fuse into one piece. This can be seen in the broken mineral structure of 13% recalcified bone (Figure 6), where seeds have only grown enough to form localized regions of mineral fusion (The small pieces of mineral). It is possible though, that mineral seed crystals actually did form a fully fused structure by 13% recalcification, but that the fused structure was not strong enough to hold its own weight and withstand the deproteinization procedure. Figure 7 shows how the thin and broken mineral pieces of 13% recalcified bone have actually fused enough to form the same shapes and resemble that of the fused continua in initial and 40% recalcified bone. Other less invasive methods – such as x-ray microscopy – may solve the fragility problem by providing an opportunity to look at the mineral phase without destroying the collagen matrix and the weak, inchoate mineral structure that exists during early phases of recalcification. Radiological techniques such as x-ray microscopy may also be beneficial in investigating 40% recalcified bone, offering insight beyond the surface of the fused mineral structure, which is all that can be seen after deproteinization. It is possible that the outside of 40% recalcified bone has a fully fused mineral

continuum, while the inside remains incomplete.

Accompanying the formation of a mineral continuum is an increase in hardness. Table 1 shows bone collagen hardness increasing as it becomes more calcified. Hardness seems to increase sigmoidally, increasing slowly at first (Seeded collagen remains flexible and soft) then speeding up and slowing down again. This makes sense theoretically: Seed crystals start out well dispersed and not connected with each other, keeping the bone collagen block flexible. During recalcification the crystals grow and quickly come into contact with each other to fuse, rapidly causing bone collagen to harden. The mineral structure hardens but remains weak at first, resulting in observations such as the 13% recalcified broken mineral structure with shapes that resemble a fused continuum (Figures 6 and 7). During later stages of calcification most of the recalcification may be going towards strengthening the mineral continuum, rather than increasing its hardness. This would explain the slowdown in hardening as the bone collagen blocks become more calcified. Ultimately however, it is difficult to draw conclusions from the subjective hardness assessment of a single handler though – more precise structural characterizations would be ideal.

While emphasis has been placed on the osteoblast-free nature of the ABS mineral growth step after seeding, it is important to note that we are only saying that osteoblasts may not be necessary for mineral growth. Osteoblasts remain present during all of osteoid calcification and may play other roles to improve or even hasten mineral formation [15] [16].

Summary and Conclusions

In this study we have attempted to approximate conditions inside a mammalian

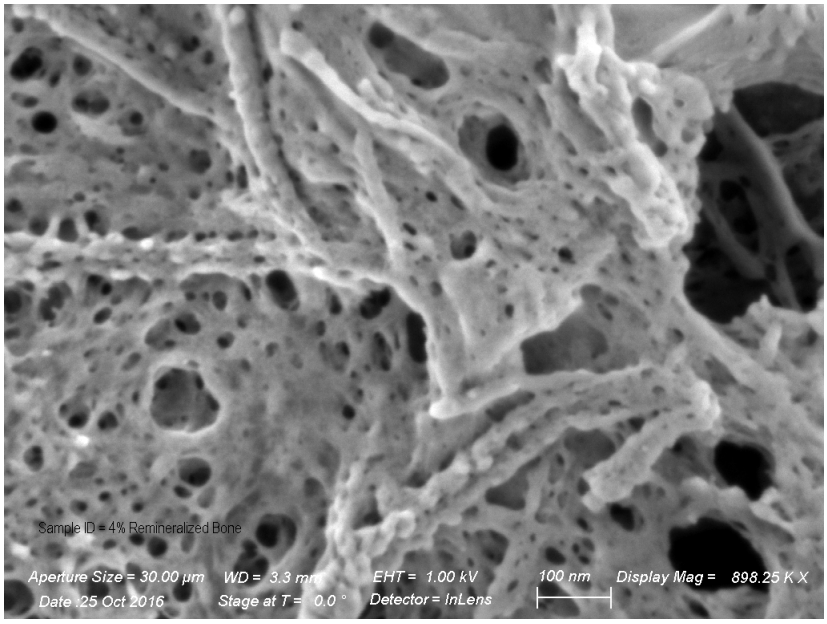
body to test whether it is possible for osteoid calcification and lamellar bone formation to occur via a seed-grow mechanism. To do this we took decalcified trabecular bone blocks (bone collagen) and attempted to recalcify them in adult bovine serum via a seed-grow process. Seeded bone collagen did ultimately recalcify in adult bovine serum, showing that it may be possible for a similar phenomenon to occur in vivo. Additionally, we have uncovered clues on how a weak, fused mineral continuum may form early during calcification, only strengthening as calcification continues.

While decalcified bone and adult bovine serum are able to model basic aspects of osteoid calcification, they are not without limitations. Adult bovine serum initially provides osteoid or bone collagen that approximates an in vivo environment, but calcium and phosphate levels quickly drop, as seeded bone collagen forms mineral. This is contrary to an in vivo environment, where homeostatic processes maintain certain levels of calcium and phosphate. Circulating blood is also known to contain high turnover factors that may influence calcification, such as pyrophosphate. Pyrophosphate is an inhibitor of mineral formation and is small enough to enter collagen matrices, but only has a half-life of half an hour in the body [24]. In our system this would quickly be depleted and not replenished until a new cycle of ABS calcification is started.

The problem of using a trabecular bone to represent osteoid is that normal osteoid calcification usually takes place on 5-10 μm thick layers of osteoid, whereas the trabeculae in the bone collagen blocks are at least 10 times as thick. The silver lining though, is that the ability for relatively thicker trabeculae to calcify by seeding and growing means that the same process is certainly possible for the thinner, 5-10 μm osteoid and its greater diffusional access to blood constituents.

Due to the limitations of our model system, an important future goal for testing the seed-grow hypothesis is the shifting to an in vivo animal model with similar bone biology to humans. Examples of this would be canine or ovine model systems [25]. Understanding the process of normal bone formation in the body has the potential to provide insight on how to treat bone diseases such as osteoporosis, which is a dysfunction of lamellar bone resorption and or formation.

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Bone mineral and collagen are intricately organized at the nanoscale. This scanning electron micrograph depicts a deproteinized bone that was remineralized to 4% of its original mineral content. The holes left behind represent the location of collagen fibers prior to deproteinization. The arrangement of holes in the remaining mineral phase reveals an extremely close association between bone collagen and mineral at the nanoscale.

REFERENCES

[1] J. E. Eastoe and B. Eastoe, “The organic constituents of mammalian compact bone,” *Biochem. J.*, vol. 57, no. 3, pp. 453–459, Jul. 1954.

[2] R. I. Martin and P. W. Brown, “Mechanical properties of hydroxyapatite formed at physiological temperature,” *J. Mater. Sci. Mater. Med.*, vol. 6, no. 3, pp. 138–143, Mar. 1995.

[3] S. M. Bowman, J. Zeind, L. J. Gibson, W. C. Hayes, and T. A. McMahon, “The tensile behavior of demineralized bovine cortical bone,” *J. Biomech.*, vol. 29, no. 11, pp. 1497–1501, Nov. 1996.

[4] L. J. Raggatt and N. C. Partridge, “Cellular and Molecular Mechanisms of Bone Remodeling,” *J. Biol. Chem.*, vol. 285, no. 33, pp. 25103–25108, Aug. 2010.

[5] L. G. Raisz, “Pathogenesis of osteoporosis: concepts, conflicts, and prospects,” *J. Clin. Invest.*, vol. 115, no. 12, pp. 3318–3325, Dec. 2005.

[6] S. L. Teitelbaum, “Bone Resorption by Osteoclasts,” *Science*, vol. 289, no. 5484, pp. 1504–1508, Sep. 2000.

[7] P. Ballanti, E. Bonucci, C. Della Rocca, S. Milani, V. Lo Cascio, and B. Imbimbo, “Bone histomorphometric reference values in 88 normal Italian subjects,” *Bone Miner.*, vol. 11, no. 2, pp. 187–197, Nov. 1990.

[8] W. A. Merz and R. K. Schenk, “A quantitative histological study on bone formation in human cancellous bone,” *Cells Tissues Organs*, vol. 76, no. 1, pp. 1–15, Jul. 1970.

[9] M. O. Agerbaek, E. F. Eriksen, J. Kragstrup, L. Mosekilde, and F. Melsen, “A reconstruction of the remodelling cycle in normal human cortical iliac bone,” *Bone Miner.*, vol. 12, no. 2, pp. 101–112, Feb. 1991.

[10] I. L. Cameron, N. J. Short, and G. D. Fullerton, “Verification of simple hydration/dehydration methods to characterize multiple water compartments on Tendon Type 1 Collagen,” *Cell Biol. Int.*, vol. 31, no. 6, pp. 531–539, Jun. 2007.

[11] D. J. Hadjidakis and I. I. Androulakis, “Bone Remodeling,” *Ann. N. Y. Acad. Sci.*, vol. 1092, no. 1, pp. 385–396, Dec. 2006.

[12] J. Kragstrup, F. Melsen, and L. Mosekilde, “Thickness of lamellae in normal human Iliac trabecular bone,” *Metab. Bone Dis. Relat. Res.*, vol. 4, no. 5, pp. 291–295, Jan. 1983.

[13] G. Bourne, *The Biochemistry and Physiology of Bone*. Elsevier, 2012.

[14] R. P. Heaney, “The bone-remodeling transient: Implications for the interpretation of clinical studies of bone mass change,” *J. Bone Miner. Res.*, vol. 9, no. 10, pp. 1515–1523, Oct. 1994.

[15] L. C. Costello, R. B. Franklin, M. A. Reynolds, and M. Chellaiah, “The Important Role of Osteoblasts and Citrate Production in Bone Formation: ‘Osteoblast Citration’ as a New Concept for an Old Relationship,” *Open Bone J.*, vol. 4, 2012.

[16] R. B. Franklin, M. Chellaiah, J. Zou, M. A. Reynolds, and L. C. Costello, “Evidence that Osteoblasts are Specialized Citrate-producing Cells that Provide the Citrate for Incorporation into the Structure of Bone,” *Open Bone J.*, vol. 6, pp. 1–7, 2014.

[17] H. Fleisch, “Role of nucleation and inhibition in calcification,” *Clin. Orthop.*, vol. 32, pp. 170–180, Feb. 1964.

[18] R. J. Staples, “Growing and Mounting Crystals Your Instrument Will Treasure,” 12-Dec-2012. [Online]. Available: https://www2.chemistry.msu.edu/facilities/crystallography/xtalgrow.pdf. [Accessed: 28-Nov-2016].

[19] W. R. Moorehead and H. G. Biggs, “2-Amino-2-methyl-1-propanol as the Alkalizing Agent in an Improved Continuous-Flow Cresolphthalein Complexone Procedure for Calcium in Serum,” *Clin. Chem.*, vol. 20, no. 11, pp. 1458–1460, Nov. 1974.

[20] S. A. Cohen and L. Sideman, “Modification of the o-cresolphthalein complexone method for determining calcium.,” *Clin. Chem.*, vol. 25, no. 8, pp. 1519–1520, Aug. 1979.

[21] P. A. Price, D. Tororian, and J. E. Lim, “Mineralization by Inhibitor Exclusion: The Calcification of Collagen with Fetuin,” *J. Biol. Chem.*, vol. 284, no. 25, pp. 17092–17101, Jun. 2009.

[22] D. Tororian, J. E. Lim, and P. A. Price, “The Size Exclusion Characteristics of Type I Collagen IMPLICATIONS FOR THE ROLE OF NONCOLLAGENOUS BONE CONSTITUENTS IN MINERALIZATION,” *J. Biol. Chem.*, vol. 282, no. 31, pp. 22437–22447, 2007.

[23] J. H. Song, “Unpublished observations.”

[24] W. C. O’Neill, K. A. Lomashvili, H. H. Malluche, M.-C. Faugere, and B. L. Riser, “Treatment with pyrophosphate inhibits uremic vascular calcification,” *Kidney Int.*, vol. 79, no. 5, pp. 512–517, Mar. 2011.

[25] A. I. Pearce, R. G. Richards, S. Milz, E. Schneider, and S. G. Pearce, “Animal models for implant biomaterial research in bone: a review,” *Eur. Cell. Mater.*, vol. 13, pp. 1–10, Mar. 2007.