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14. ABSTRACT Our primary objective is to demonstrate approaches to create mechanically robust, patient-customized biomaterials for large, load-bearing maxillofacial bone defects. To do this, we are developing approaches to integrate a biomolecule decorated collagen scaffold with micro-scale porosity into a mechanically-robust polymeric frame generated via 3D-printing with macro-porosity. Through the second year of this project we have completed the design, fabrication, as well as mechanical and in vitro osteogenesis testing of multiple elements of the final composite biomaterial design. We identified a PLA-collagen composite that could support robust mesenchymal stem cell (MSC) viability, osteogenic differentiation, and new mineral synthesis while rendering it shape-fitting to improve conformal contact with the wound margin. We have advanced multiple strategies to incorporate biomolecular signals into the collagen scaffold via transient sequestration, covalent attachment, and through included zinc ions that enhance osteogenic activity. Ongoing efforts are completing biomolecule incorporation and release experiments to facilitate long-term (>7 day) biomolecule bioavailability via mineral-based factor sequestration chemistries. Our efforts are essential for our goal to identify a shelf-stable, patient customizable biomaterial that can be seeded with autologous MSCs to regenerate large CMF bone defects.					
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INTRODUCTION:

Severe craniomaxillofacial (CMF) injuries are prevalent after a wide range of acute and chronic injuries, are typically large in size, and are characterized by significant loss of hard and soft tissue. Our primary objective is to demonstrate a novel approach for creating mechanically robust, patient-customized biomaterials for large, load-bearing maxillofacial bone defects. We address a fundamental bottleneck in CMF biomaterial design. Constructs must balance very real considerations regarding mechanical competence and load bearing, the need to fit complex defect geometries unique to each patient, and biotransport of nutrients within the construct during healing. To do this, we are developing approaches to integrate a biomolecule decorated collagen scaffold with micro-scale porosity into a mechanically-robust polymeric frame generated via 3D-printing with macro-porosity. We are first validating the osteogenic potential of growth factor decorated composite (Aim 1). We are evaluating the influence of composite structural properties on mesenchymal stem cell (MSC) osteogenesis in vitro and will subsequently establish whether, and to what extent, selective incorporation and release of the growth factors BMP-2/VEGF, the mineral zinc, or modifications to scaffold glycosaminoglycan (GAG) content enhances osteogenesis. Next, we will examine the quality and kinetics of mandible bone regeneration using the composite in critically-sized mandibular ramus defects in the Yorkshire pig (Aim 2). The larger goal of this program is to identify a shelf-stable, patient customizable biomaterial that can be seeded with autologous MSCs intraoperatively and immediately implanted in order to regenerate large, load-bearing CMF bone defects.

KEYWORDS:

Biomaterial, composite, collagen, growth factor release, osteogenesis

OVERALL PROJECT SUMMARY:

Through the final year of this project, we have completed all tasks and milestones associated with this project, which was to validate the osteogenic potential of the biomolecule decorated collagen composites via tiered in vitro and in vivo assays. Over the prior year, we completed both a short-term (6 week) and long-term (6 month) craniofacial bone regeneration study using a porcine mandible defect model.

MAJOR TASK 1: Composite Fabrication

The primary goals associated with Major Task 1 are:

- Fabricate PCL cages for collagen-PCL composites
- Fabricate library of collagen-PCL composites and growth factor modified composites for in vitro testing

Key Milestones associated with Major Task 1 are:

- Biophysical characterization of mineralized collagen scaffolds and PCL-collagen composites
- Biophysical and functional characterization of growth factor loading/elution

MAJOR TASK 2: In vitro osteogenesis assays

The primary goals associated with Major Task 2 are:

- Assess mechanisms by which the mineralized collagen scaffold accelerate MSC osteogenic differentiation and matrix biosynthesis.

Key Milestones associated with Major Task 2 are:

- Identify the degree to which BMP-2, VEGF, and growth factor sequestering chemistries incorporated within the collagen scaffolds increase MSC osteogenic differentiation.

Summary of Results, Progress and Accomplishments with Discussion:

PRIOR WORK (reported in YR2 annual report). First generation composite biomaterials for CMF repair. A significant challenge to improving the quality and speed of craniomaxillofacial bone regeneration are competing design requirements for a biomaterial platform: porosity required for cell recruitment and adequate biotransport; mechanical strength that is significantly reduced by the inclusion of pores; shape-fitting to improve conformal contact and osseointegration between the implant and the defect. With USMRMC funding, we developed a new class of collagen composite biomaterial. We leveraged 3D printing tools to create macro-scale poly (lactic acid) (PLA) reinforcement frames that can be integrated into the collagen suspension prior to lyophilization, resulting in a multiscale scaffold-fiber composite. A critical advance associated with this work was demonstrating an approach to render the composite shape-fitting to address an unmet clinical need: the need for close conformal contact between biomaterial implant and the surrounding wound site. We showed selective removal of circumferential fiber segments from the PLA frame could yield a composite that was deformable radially yet retained sufficient spring-back capacity to increase the required push-out force. These findings confirm that the addition of even small volume fractions (~10% v/v) of polymeric mechanical reinforcement are sufficient to increase composite mechanical strength and address

the current translational limitation of the mineralized collagen scaffold (compressive moduli < 1MPa). We completed biophysical and vitro MSC osteogenic activity characterization [1].

PRIOR WORK (reported in YR3 annual report). Zinc-modified scaffolds promote scaffold osteogenic activity. Implant osteoinduction and subsequent osteogenic activity are critical events that need improvement for regenerative healing of large craniofacial bone defects. While preliminary data was reported in the Year 2 Annual report, in the past year we successfully completed our study of incorporation of zinc ions into the mineralized collagen scaffold to accelerate MSC osteogenesis. Zinc is an essential trace element in skeletal tissue and bone, with soluble zinc being shown to promote osteogenic differentiation of porcine adipose derived stem cells. We augmented the mineral content of a class of mineralized collagen scaffolds under development for craniomaxillofacial bone regeneration via the inclusion of zinc ions to promote osteogenesis in vitro. Zinc sulfate was added to a mineralized collagen-glycosaminoglycan precursor suspension then freeze dried to form a porous biomaterial. We reported biophysical parameters of zinc functionalized scaffolds via imaging (scanning electron [2]microscopy), mechanical testing (compression), and compositional (X-ray diffraction, inductively coupled plasma mass spectrometry) analyses. Zinc-functionalized scaffolds display morphological changes to the mineral phase and altered elastic modulus without substantially altering the composition of the brushite phase or removing the micro-scale pore morphology of the scaffold. These scaffolds also display zinc release kinetics on the order of days to weeks and promote successful growth and pro-osteogenic capacity of porcine adipose derived stem cells cultured within these zinc scaffolds. Taken together, we believe that zinc functionalized scaffolds provide a unique platform to explore strategies to improve in vivo osteogenesis in craniomaxillofacial bone injuries models [3].

Mineralized collagen scaffolds fabricated with amniotic membrane matrix increase osteogenesis under inflammatory conditions. Defects in craniofacial bones occur congenitally, after high-energy impacts, and during the course of treatment for stroke and cancer; improved regenerating healing likely requires addressing challenges associated with the inflammatory environment surrounding the injury. We have adapted the mineralized collagen scaffold under development as part of this project, already capable of supporting significant osteogenic differentiation and matrix biosynthesis in the absence of osteogenic media or supplemental proteins, to include amniotic membrane matrix derived from placentas (Fig. 1). We report increased mechanical properties of a mineralized collagen–amnion scaffold and investigated osteogenic differentiation and mineral deposition of porcine adipose-derived stem cells within these scaffolds as a function of inflammatory challenge. Incorporation of amniotic membrane matrix promotes osteogenesis similarly to un-modified mineralized collagen scaffolds, and increases in mineralized collagen–amnion scaffolds under inflammatory challenge. Together, these findings suggest that a mineralized collagen–amnion scaffold may provide a beneficial environment to aid craniomaxillofacial bone repair, especially in the course of defects presenting significant inflammatory complications. See appendix: [2].

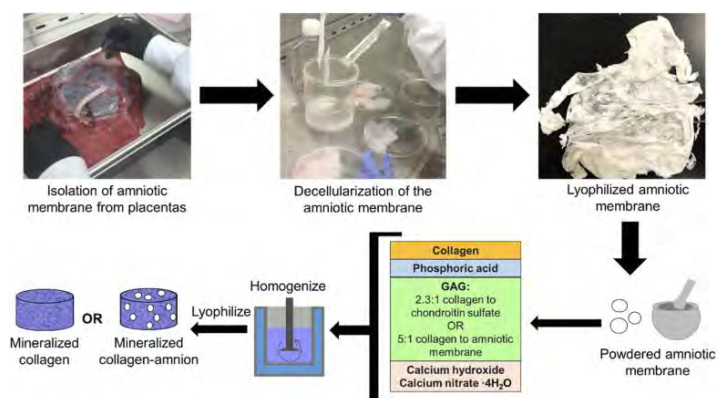


Fig. 1. Isolation of amniotic membrane from placentas and synthesis of mineralized collagen and mineralized collagen–amnion scaffolds.

Anisotropic mineralized collagen scaffolds accelerate osteogenic response in a glycosaminoglycan-dependent fashion. Regeneration of critically-sized craniofacial bone defects requires a template to promote cell activity and bone remodeling. However, induced regeneration becomes more challenging with increasing defect size. Methods of repair using allografts and autografts have inconsistent results, attributed to age-related regenerative capabilities of bone. We adapted the mineralized collagen scaffold under development as part of this project seeking scaffold technologies to promote craniomaxillofacial bone regeneration as an alternative to repair. We hypothesized modifying the pore anisotropy and glycosaminoglycan content of the scaffold will improve cell migration, viability, and subsequent bone formation (Fig. 2). Using anisotropic and isotropic scaffold variants, we first tested the role of pore orientation on human mesenchymal stem cell (MSC) activity. We subsequently explored the role of glycosaminoglycan content, notably chondroitin-6-sulfate, chondroitin-4-sulfate, and heparin sulfate on mineralization. We found that while short term MSC migration and activity was not affected by pore orientation, increased bone mineral synthesis was observed in anisotropic scaffolds. Further, while scaffold glycosaminoglycan content did not impact cell viability, heparin sulfate and chondroitin-6-sulfate containing variants increased mineral formation at the late stage of in vitro culture, respectively. Overall, these findings show scaffold microstructural and proteoglycan modifications represent a powerful tool to improve MSC osteogenic activity. See appendix: [4].

Stiffness of Nanoparticulate Mineralized Collagen Scaffolds Triggers Osteogenesis via Mechanotransduction and Canonical Wnt Signaling. As part of our further optimization of scaffold biophysical properties to support craniofacial bone regeneration, we examined the role of carbodiimide crosslinking induced stiffening of the mineralized scaffold on MSC osteogenic activity. We reported that the use of carbodiimide crosslinking increases scaffold elastic modulus 10-fold. Moreover, we examined two aspects of MSC osteogenic differentiation in response to scaffold stiffening. Both crosslinked and non-crosslinked scaffolds are capable of autogenously activating the canonical BMPR signaling pathway with phosphorylation of Smad1/5. Interestingly, human mesenchymal stem cells cultured on crosslinked scaffolds display significantly elevated expression of the major mechanotransduction mediators YAP and TAZ expression, coincident with β -catenin activation in the canonical Wnt signaling pathway. Inhibiting YAP/TAZ activation reduces osteogenic expression, mineralization, and β -catenin activation in crosslinked scaffolds moreso than non-crosslinked. These results indicate that increasing scaffold stiffness via carbodiimide crosslinking induces osteogenic differentiation via the mechanotransduction mediators YAP/TAZ and the canonical Wnt signaling pathway, whereas the canonical BMPR signaling pathway is activated independent of scaffold stiffness. This adds an important observation regarding alterations to the

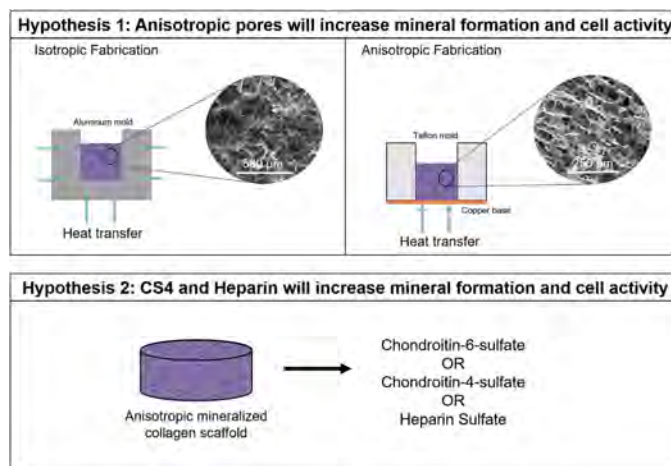


Fig. 2. Hypothesis 1: anisotropic pores will have greater mineral formation and cell activity than isotropic pores. The different molds used during freeze drying to fabricate isotropic and anisotropic mineralized collagen scaffolds is demonstrated. Hypothesis 2: chondroitin-4-sulfate and heparin sulfate will have greater mineral formation and cell activity than scaffolds containing chondroitin-6-sulfate. Anisotropic scaffolds containing the same amount of chondroitin-6-sulfate, chondroitin-4-sulfate, or heparin sulfate are compared.

mineralized collagen scaffold to maximally support MSC osteogenesis. See appendix: [5].

Sequential sequestrations increase the incorporation and retention of multiple growth factors in mineralized collagen scaffolds. Over the past year we completed analysis of, and successfully published a manuscript describing, the use of non-covalent growth factor sequestration methods to improve the bioactivity of our mineralized collagen scaffold. This is a major element of the proposed efforts in this project and has been a project that took almost two years to complete. Here, growth factor signaling has the potential to coordinate the behavior of multiple cell types following an injury, and effective alteration of growth factor availability within a biomaterial can be critical for accelerating bone healing. During this project we have optimized the design parameter of a mineralized collagen scaffolds to facilitate cell invasion and MSC osteogenesis. Here we describe the use of modified simulated body fluid treatments to enable sequential sequestration of bone morphogenic protein 2 and vascular endothelial growth factor into the mineral phase of these mineralized collagen scaffolds (Fig. 3). This approach is unlike traditional crosslinking based methods for incorporating growth factors; we have now showed as part of this project optimization of scaffold structure (pore anisotropy [4]), scaffold composition (glycosaminoglycan content [4]; incorporation of amnionic membrane[2]), and crosslinking[5] to support beneficial MSC osteogenic activity. Hence, incorporation of growth factors must be performed in a manner orthogonal to those efforts. Here we show that sequential exposure to biomolecules of interest and simulated body fluid can be used to sequester 60–90% of growth factor from solution into the mineral phase without additional crosslinking treatments. This approach allows high levels of retention for both individual growth factors (>94%) or multiple growth factors (>88%) that can be layered into the material via sequential sequestration steps. Sequentially sequestering growth factors allows prolonged release of growth factors in vitro (>94%) and suggests the potential to improve healing of large-scale bone injury models in vivo. Future work will utilize this sequestration method to induce cellular activities critical to bone healing such as vessel formation and cell migration. See appendix: [6].

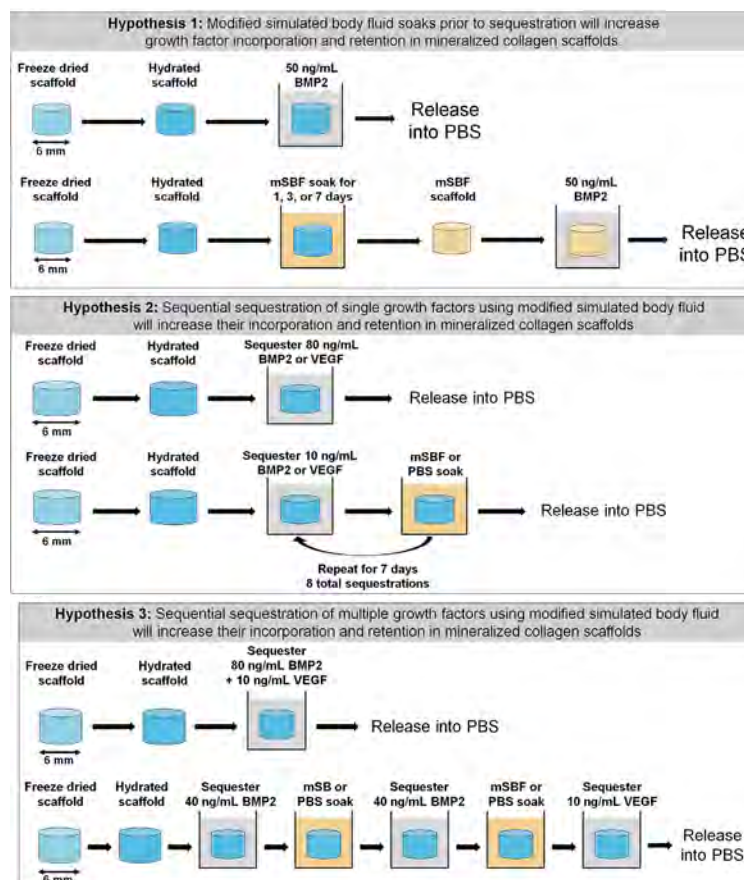


Fig. 3. Hypothesis 1: Growth factor sequestration with varying modified simulated body fluid (mSBF) treatments. Hypothesis 2: Sequential sequestration of individual growth factors (BMP2 or VEGF). Hypothesis 3: Sequential sequestration of multiple growth factors (BMP2 and VEGF) versus sequentially sequestered BMP2 then VEGF.

Inclusion of a 3D-printed mesh improves mechanical and osteogenic performance of a mineralized collagen scaffold. During this project we have reported on the concept of incorporating polymer-based reinforcement meshes into the mineralized collagen scaffold to increase the mechanical performance of the resulting composite. In work recently submitted[7] we showed the degradation byproducts and acidic release from the printed polymer structures have limited negative impact on the viability of mesenchymal stem cells but may be chosen to actively promote osteogenic activity. Inclusion of a mesh (Fig. 5) formed from Hyperelastic Bone™ bioinks generates a reinforced composite with significantly improved mechanical performance (elastic modulus, push-out strength). Composites formed from the mineralized collagen scaffold and either Hyperelastic Bone supported human bone-marrow derived mesenchymal stem cell osteogenesis and mineral biosynthesis. Strikingly, composites reinforced with Hyperelastic Bone mesh elicited significantly increased secretion of osteoprotegerin, a soluble glycoprotein and endogenous inhibitor of osteoclast activity. These results suggest that architected meshes can be integrated into collagen scaffolds to boost mechanical performance and actively instruct cell processes that aid osteogenicity; specifically, secretion of a factor crucial to inhibiting osteoclast-mediated bone resorption. Future work beyond the scope of this project will focus on further adapting the polymer mesh architecture to confer improved shape-fitting capacity as well as to investigate the role of polymer reinforcement on MSC-osteoclast interactions as a means to increase regenerative potential.

Adapting the design of conformal fitting polymeric reinforcement cages to meet translational challenges.

During Year 3 of this project, we reported [1] a polymer fiber reinforcement strategy for the mineralized collagen scaffold. As stiffness requirements for confined CMF bone defects

are lower (5-10 MPa[8]) than for segmental bone defects, we prioritized biomaterial innovations to improve conformal contact with the defect margin. The initial composite used a PLA-fiber array to create conformal fitting design elements for our scaffold. In the past year we adapted the design of the reinforcing cage for the porcine CMF defect model. This requires a more robust PCL-based bioink for comparison to results reported outside of this project that used a high-density PCL reinforcing strategy. We have fabricated prototype PCL reinforcement cages with conformal-fitting geometries and received ACURO approval for animal studies of PCL-

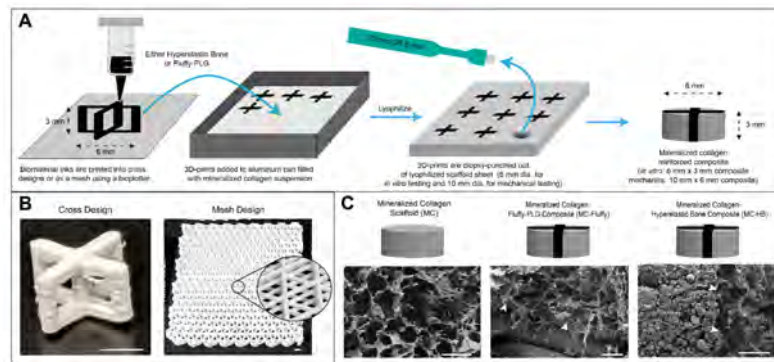


Fig. 5. Fabrication of composites used in vitro and for mechanical testing. (A) Hyperelastic Bone and Fluffy-PLG were 3D printed in “cross designs” for in vitro testing or as a mesh for mechanical testing. Cross prints were printed individually and added to a continuous mineralized collagen suspension in an aluminum mold. After the addition of multiple prints, the entire mold was freeze-dried and a 6 mm biopsy punch was used to remove scaffold and 3D-print together. Mesh reinforced composites were fabricated by adding the entire mesh to the aluminum mold with mineralized collagen suspension and biopsy punching 10 mm diameter composites after lyophilization. Mesh 3D-prints were printed with either 2 mm or 3 mm line spacing. (B) Images of cross design and mesh 3D-prints, scale bar represents 3 mm. (C) SEM images of mineralized collagen scaffold and cross design reinforced composites and acronyms used to represent the groups in the study. Scale bar represents 150 μ m and white arrows indicate interface between the mineralized collagen and 3D-printed reinforcing structures.

reinforced mineralized collagen scaffolds in a porcine mandible defect. We have developed proof-of-concept for a novel composite based on the mechanical performance of low-density Voronoi foams (Fig. 6). These open cell foams pack to fill space and their random nature (absence of regularity) affords a simple approach to define mechanical performance independent of foam geometry.[9,10] Under load, the fibers that define the pores of these foams bend elastically during the first ~10% applied strain then buckle plastically.[9] Mechanical performance of these meshes is controlled by overall relative density (ρ^*/ρ_s ; ρ^* , density of the porous material; ρ_s , density of the solid phase) not pore geometry, making them ideal for maintaining mechanical properties despite being cut/shaped. So rather than requiring a global optimization process, Voronoi foams can be rapidly generated to meet defined mechanical benchmarks.

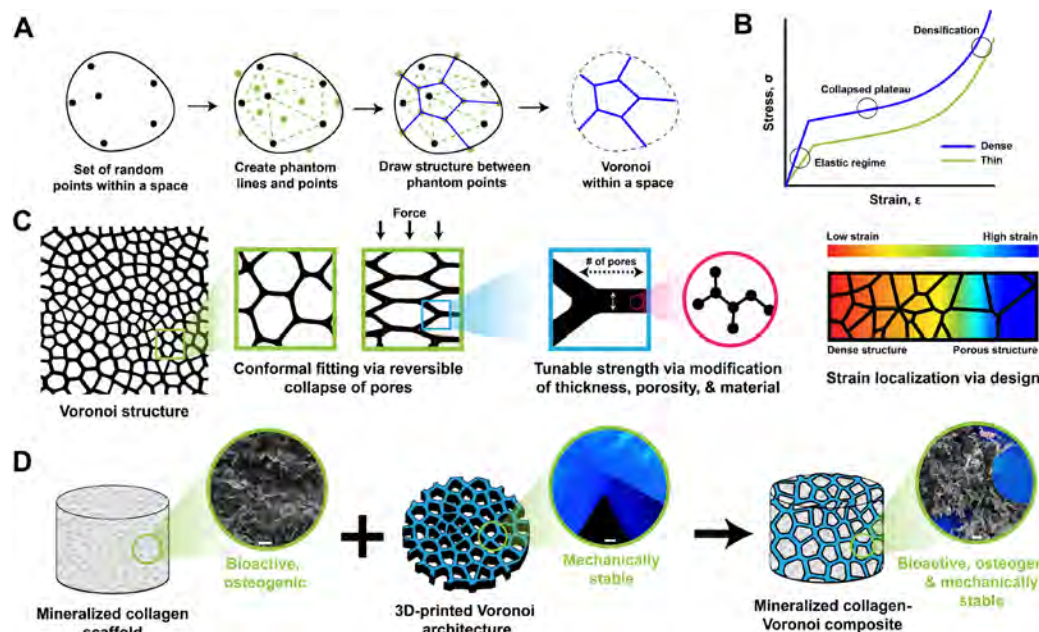


Fig. 6. Tough and tunable Voronoi structures. (A) Creating a Voronoi structure within any space. First, a set of “seed” points are added to the space. Then phantom lines (green) are drawn between these points, with phantom points (green) in the middle of these areas. Finally, the lines of the Voronoi structure (blue) are drawn between the phantom lines connecting the phantom points. (B) Representative stress-strain curve of foams, such as Voronoi structures. These structures are characterized by a linear elastic regime, a collapsed plateau, and densification. Increasing the density of Voronoi structures increases the modulus (elastic regime). (C) Voronoi structures can be added to any size or shape design and can achieve conformal fitting by the reversible collapse of pores. Additionally, the thickness of the individual struts in the architecture and the material it is comprised of can lead to tunable strength and elasticity. Finally, strain can easily be localized by printing these designs with regions of variable porosity or density of material. (D) The goal of this study was to incorporate tunable Voronoi 3D-printed architectures into mineralized collagen scaffolds for use in bone repair. Mineralized collagen scaffolds offer excellent bioactivity and osteogenic properties due to the mineral, glycosaminoglycans, collagen, and porous nature of this material, however, this porosity also lends to these being very soft structures and more difficult to handle in bone repair situations. 3D-printed Voronoi structures offer a mechanically stable material with tunable mechanics, however, the macro-scale porosity and print material may not be as osteogenic as mineralized collagen scaffolds. Combined together, mineralized collagen and 3D-printed Voronoi structures have the ability to promote osteogenesis while also maintaining strength. Scale bar represents 100 μm .

The mechanical performance of architected Voronoi foams is predictably dependent on print parameters (porosity, strut thickness) that can be defined by generative design algorithms (Fig. 7). As predicted, the porosity and thickness of printed Voronoi structures altered the Young's Modulus, all decreasing the Young's Modulus as density decreased. Due to the random structure of Voronoi designs, these by nature should be isotropic; however, in all of our tests we noticed some degree of anisotropy, as two or more sides had different moduli. This could be attributed to the small size of these designs and the large point spacing (i.e. 10 mm cubes with 4 mm point spacing). We hypothesize that a larger design, such as sheets of Voronoi structures, would result in a more isotropic structure, and CMF defects are typically 25 mm or larger in size. Voronoi computation models used to relate moduli to predictive equations have used a minimum of 27 cells to accurately assess isotropy 66, suggesting the differences in modulus may be due to an insufficient number of pores in that axis of compression.

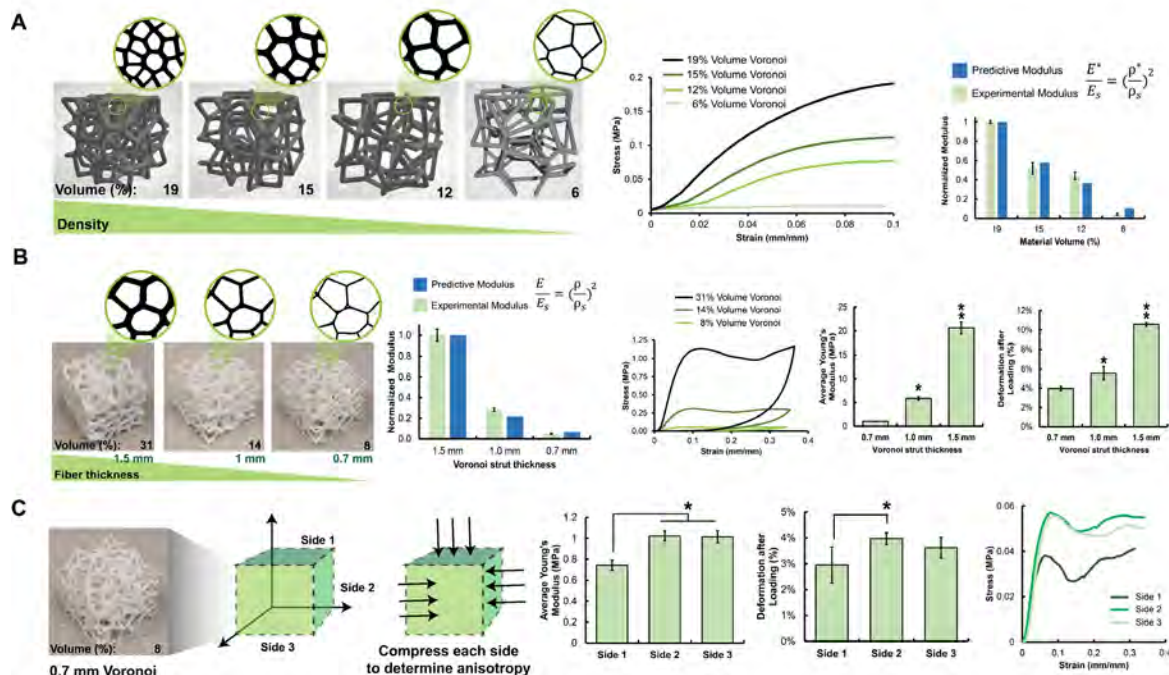


Fig. 7. Tuning stiffness through Voronoi density and material thickness. (A) Four Voronoi designs (10 mm cubes) of different porosities altered by changing the architecture were fabricated by 3D-printing photopolymerized resin. Designs were compressed and the stress-strain profiles were recorded, and as the volume % material of the design increased, the stiffness increased. The modulus of the four designs was compared to a predictive modulus for Voronoi architectures. (B) Three of the same Voronoi design (25 mm cubes) with different material thicknesses were 3D-printed using polycaprolactone. The modulus of these designs were tested against a predictive modulus for Voronoi architectures. Samples were loaded and unloaded compressively and the stress-strain profiles were recorded. The average modulus and deformation after loading increased with increasing strut thickness. ** indicates the 1.5 mm group was significantly ($p < 0.05$) greater than all other groups. * indicates the 1.0 mm group was significantly ($p < 0.05$) greater than all other groups. (C) Analyzing anisotropy in 0.7 mm thickness Voronoi designs printed with polycaprolactone and 8% volume material (25 mm cubes). Each of the sides of one Voronoi design (x, y, z axis) were loaded and unloaded under compression and the modulus and deformation after loading were examined. * indicates which group(s) were significantly ($p < 0.05$) greater than another group. Differences in the modulus between sides of the same design can indicate anisotropy. Data expressed as average $\pm$ standard deviation ($n=6$).

Local changes to Voronoi mesh architecture enable localized control over composite deformation (Fig. 8). We designed a biphasic 3D-print with a porous and dense region and were able to successfully combine this with mineralized collagen scaffolds to create a composite material, and further compared this to a composite with only one reinforced phase (porous). The compression of the biphasic 3D-print and

composite demonstrated stress-strain profiles of two different density materials, and DIC analysis demonstrated that strain is concentrated at the porous region. Compared to Voronoi composites with one phase, stress localization was randomly throughout the material. Upon comparing to the predictive modulus, this was fairly accurate for the dense structure, but not as accurate for the porous portion, most likely due to the analysis of the stress-strain curve portions and these combined structures leading to an altered moduli.

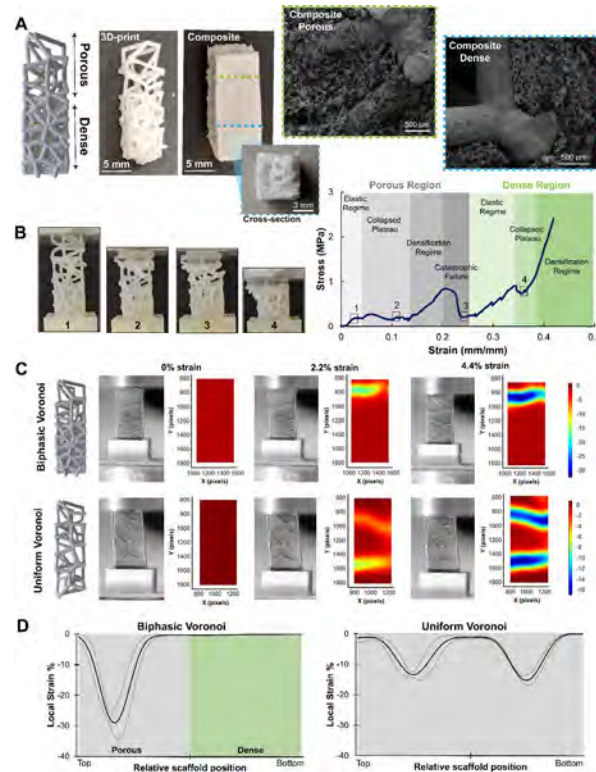


Fig. 8. Localizing strain through biphasic Voronoi design. (A) Biphasic Voronoi designs were 3D-printed with photopolymerized resin into designs with a distinctly porous region and dense region. These were combined with mineralized collagen scaffolds to create a composite material. SEM images demonstrate the integration of the 3D-print with the surrounding mineralized collagen in the dense region (blue) and porous region (green). (B) Biphasic Voronoi 3D-prints were compressed and the stress-strain profile was recorded. The stress-strain profile was comprised of two different curves, whereas the porous region first compressed until failure (grey), and then the dense region was compressed (green), outlined on the stress-strain profile with matching images of the 3D-print under compression. (C) Biphasic Voronoi and mineralized collagen composites were compared to uniform Voronoi mineralized collagen composites, with the uniform region being the same throughout as the porous region of the biphasic Voronoi. These were compared under compression and further Digital Image Correlation to map strain regions and create contour plots. At 0%, 2.2%, and 4.4% global applied strain, images of the composite are alongside representative strain localization images, with blue representing the greatest strain. (D) Line scans of the strain on biphasic Voronoi composite and uniform Voronoi composite were created from Digital Image Correlation data. Average strain at a global applied 4.4% strain was plotted as a function of position for each sample, with the average (black line) and standard deviation (interval contained by gray lines) of strain magnitude along the entire length of each scaffold. The porous region is demonstrated by a grey background and the dense region with green (n=6).

Provision Patent. Concepts described to the above described work were also successfully filed as a Provisional Patent applications (U.S. Provisional Patent Application No. 63/440,325, 1/20/2023; appended to this report).

In vivo assessment of craniofacial bone regeneration capacity using a porcine mandible defect model. Severe craniomaxillofacial (CMF) injuries are prevalent after a wide range of acute and chronic injuries, are typically large in size, and are characterized by significant loss of hard and soft tissue. Our primary objective is to demonstrate a novel approach for creating mechanically robust, patient-customized biomaterials for large, load-bearing maxillofacial bone defects. We developed a method to integrate a bioactive, growth factor decorated collagen scaffold with micro-scale porosity into a mechanically-robust polycaprolactone (PCL) cage with macro-porosity. We have chosen the pig as the animal model for this study. The Yorkshire pig is an appropriate pre-clinical model as the porcine mandible has similar biomechanical and anatomic properties to the human mandible. We assessed the regenerative potential of the collagen-PCL composite using subcritical and critically-sized mandibular ramus defects in the Yorkshire pig. This large animal model represents an essential step towards clinical trials that extends beyond our previous efforts using small animal models (e.g., rabbit). We have completed two distinct in vivo regeneration trials: **1)** healing processes in a sub-critical mandible bone defect; **2)** regenerative healing of a critical-sized mandible defect (**Fig. 9**).

In vivo study 1: COMPLETED

Goal: Test the bone ingrowth into a composite formed as a growth factor decorated mineralized collagen scaffold with micro-scale porosity containing a mechanically-robust polycaprolactone (PCL) mesh with macro-porosity design to improve implant-defect conformal fitting. Composite specifications and growth factor loading previously[1,6] optimized during this project.

Defect size: 10 mm diameter, 10mm deep cylindrical mandibular defects

Healing period: 6 weeks

Implants tested: Total 36 (6 Yorkshire pigs; 3 defects per hemi-mandible; randomly distributed)

- 12 implants: non-modified mineralized collagen scaffolds
- 12 implants: collagen scaffold + PCL mesh composite
- 12 implants: collagen scaffold + PCL mesh composite + BMP2 (75ng)/VEGF (15ug)

In vivo study 2: COMPLETED

Goal: Test the regenerative capacity of composite formed as a growth factor decorated mineralized collagen scaffold with micro-scale porosity containing a mechanically-robust polycaprolactone (PCL) mesh with macro-porosity design to improve implant-defect conformal fitting. Increased growth factor dose per scaffold was due to increased volume of larger defects used in this study.

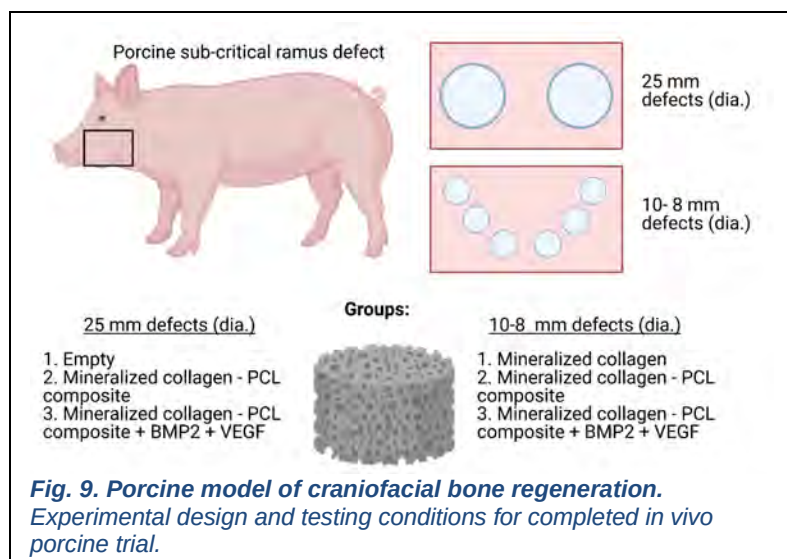
Defect size: 25 mm diameter; 10mm deep cylindrical mandibular defects

Healing period: 6 months

Implants tested: Total 36 (18 Yorkshire pigs; 1 defect per hemi-mandible; randomly distributed)

- 12 implants: empty defect (negative control to confirm critical sized nature of defect)
- 12 implants: collagen scaffold + PCL mesh composite
- 12 implants: collagen scaffold + PCL mesh composite + BMP2 (350ng)/VEGF (70ug)

Assessment. All in vivo trials have ended and all implants have been recovered. Anecdotally, inclusion of a PCL mesh inside the mineralized



collagen scaffold to form a shape-fitting composite significantly increased surgical handleability and ease of implantation (per veterinary surgeon who as responsible for implantation). We are completing analysis of short-term and long term bone healing via μ CT (mineral and bone ingrowth); histology via H&E (bone tissue formation) and immunohistochemistry (osteoprogenitor invasion) on demineralized, embedded, and sectioned tissue specimen; biomechanics (via push-out test[1,11]) to quantify integration of the implants with surrounding native bone.

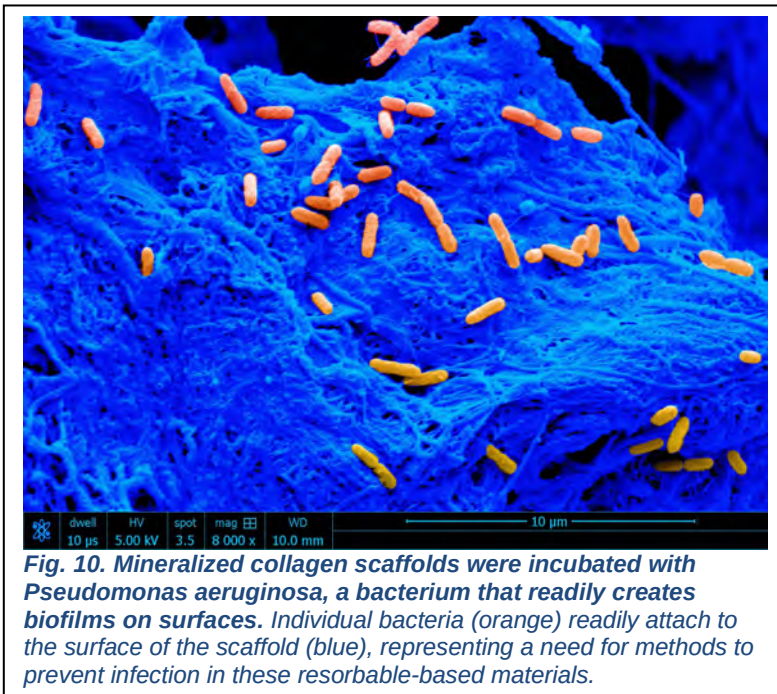
Inspired by this project (not completed using project funding): One of the trainees associated with this project wrote an option article about needs to develop new avenues to support large scale bone regeneration. This article calls for future efforts to integrate findings related to the biology of the growth plate with biomaterial design strategies. Concepts described in the manuscript were separately submitted to our campus Office of Technology Management as an intellectual property disclosure (appended to this report):

Growing pains: the need for engineered platforms to study growth plate biology [12]. Growth plates, or physis, are highly specialized cartilage tissues responsible for longitudinal bone growth in children and adolescents. Chondrocytes that reside in growth plates are organized into three distinct zones essential for proper function. Modeling key features of growth plates may provide an avenue to develop advanced tissue engineering strategies and perspectives for cartilage and bone regenerative medicine applications and a platform to study processes linked to disease progression. In this review, a brief introduction of the growth plates and their role in skeletal development is first provided. Injuries and diseases of the growth plates as well as physiological and pathological mechanisms associated with remodeling and disease progression are discussed. Growth plate biology, namely, its architecture and extracellular matrix organization, resident cell types, and growth factor signaling are then focused. Next, opportunities and challenges for developing 3D biomaterial models to study aspects of growth plate biology and disease *in vitro* are discussed. Finally, opportunities for increasingly sophisticated *in vitro* biomaterial models of the growth plate to study spatiotemporal aspects of growth plate remodeling, to investigate multicellular signaling underlying growth plate biology, and to develop platforms that address key roadblocks to *in vivo* musculoskeletal tissue engineering applications are described.

Inspired by this project (not completed using project funding): While preparing for the large-scale *in vivo* trials completed over the past year, our investigative team took seriously strategies to reduce the likelihood of implant infection. As a result, we embarked on an *in vitro* project to assess the likelihood of bacterial growth within the collagen scaffold under development by this project:

Evaluation of bacterial attachment on mineralized collagen scaffolds and addition of manuka honey to increase mesenchymal stem cell osteogenesis [13]. The design of biomaterials to regenerate bone is likely to increasingly require modifications that reduce bacterial attachment and biofilm formation as infection during wound regeneration can significantly impede tissue repair and typically requires surgical intervention to restart the healing process. Infection modeling and prevention is often performed on metal-alloy materials for bone repair applications. However, current research focuses on repair of craniomaxillofacial, or skull and jaw bone, defects focus on the use of bioresorbable implants. Infection characteristics of these materials are poorly understood, and surface properties and chemistries are largely different from metal alloys. Mineralized collagen scaffolds, or porous sponges formed from calcium phosphate mineral and bovine collagen, can be used to repair bone defects. We reported the ability of a mineralized collagen biomaterial to natively resist infection and examine how the addition of manuka honey, previously identified as an antimicrobial agent, affects gram positive and negative bacterial colonization and mesenchymal stem cell osteogenesis and vasculature formation. Mineralized collagen scaffolds were incubated with *Pseudomonas aeruginosa*, a bacterium that readily creates biofilms on surfaces. Individual bacteria (orange) readily attach to the surface of the scaffold (blue), representing a need for methods to prevent infection in these resorbable-based materials (**Fig. 10**). Our results suggest bioresorbable scaffolds may create an environment conducive to bacterial growth, and potential trade-offs exist for the incorporation of low levels of honey

in scaffolds to increase osteogenic potential of osteoprogenitors while high-levels of honey may be sufficient to reduce gram positive or negative bacteria activity but at the cost of reduced osteogenesis.



KEY RESEARCH ACCOMPLISHMENTS (cumulative):

The overall goal of this project is to develop a novel composite collagen biomaterial to improve the quality and speed of craniomaxillofacial bone regeneration. The major elements of the project are to demonstrate design modifications to an existing mineralized collagen scaffold in order to:

- Create a polymer-scaffold composite that exhibits improved compressive strength (vs. the scaffold alone) as well as conformal fitting capacity to aid the composite to fit Against the wound margins of irregular defect geometries.
- Demonstrate material and cytokine delivery innovations that accelerate cell infiltration into the scaffold as well as subsequent osteogenic differentiation and angiogenic activity.
- Validate improvements to this scaffold via assessment of the quality and kinetics of new bone formation in a porcine mandibular defect model.

Key accomplishments to date to meet these challenges:

- Demonstrating multi-scale composite reinforcement paradigm for mineralized collagen scaffolds to address acute clinical needs for biomaterials with improved macro-scale compressive strength and conformal fitting within the defect margins [1].
- Confirming incorporation of a polymeric reinforcement frame does not negatively influence the osteogenic capabilities of porcine adipose stem cells within the multi-scale composite [1].
 - NOTE: This finding extends beyond previous publications that examined the activity of human and rabbit bone marrow derived MSCs in the mineralized collagen scaffold alone [14,15] or that only confirmed adMSC viability in one design variant of a collagen-PCL composite [16].
- Developing and characterizing a novel zinc-functionalized variant of the mineralized collagen scaffold at the core of this CMF bone regeneration project. We confirmed zinc can be incorporated into and released from the scaffold microstructure. We showed incorporation of zinc improved scaffold mechanical properties, and that while zinc incorporation alters microscale mineral morphology of the scaffold it does not affect the Brushite phase of the calcium phosphate deposits. We showed zinc-functionalized scaffolds accelerate adMSC proliferation and metabolic health while maintaining osteogenic differentiation and mineral deposition capacity [3].
- In addition to long standing analysis of MSC-osteogenesis within mineralized collagen scaffolds, we have showed the mineralized collagen scaffold may also alter activity of osteoclasts [17,18], and motivates ongoing efforts to better investigate scaffold-osteoclast-MSC interactions in the context of matrix remodeling.
- We demonstrated the use of cyclodextrin-based transient sequestration of growth factors within the scaffold could enhance BMP-2 mediated osteogenic differentiation [19]. While short term growth factor retention may accelerate cell recruitment, cyclodextrin studies are now motivating current exploration of mineral-linked growth factor retention as an alternative means to delivery growth factors within the collagen scaffold.
- We demonstrated the use of nanostructured mineral-based sequestration of growth factors within the scaffold to enhance the bioavailability of BMP-2 and VEGF (manuscript in preparation). Ongoing efforts will: 1) define the long-term biological efficacy of growth factor retention; and 2) identify the VEGF loading paradigm that efficiently activates

vascular cell activity. These efforts are expected to be early in Year 4 of this project and are necessary prior to in vivo testing of a final scaffold composite in a porcine mandibular defect.

- We completed design and manufacture of a novel PCL polymeric reinforcement cage. The original PCL cage structure detailed in the motivation for this project contained high (>30% vol/vol) polymer. We previously reported a fiber-based design to improve strength and conformal fitting of a composite, but this relied on PLA-fibers. We have completed adaptation of the PLA design to the additive manufacturing technology required to fabricate PCL structures. This PCL design has the dual capacity to improve the compressive strength of scaffold-PCL composites while also providing the opportunity to enhance conformal-fitting capacity of the resulting implants (manuscript in preparation).
- We have adapted the structure of the mineralized collagen scaffold, creating a new class of anisotropic mineralized collagen scaffolds. We have defined the role of scaffold anisotropy (the inclusion of aligned tracks of pores) to aid MSC osteogenesis and infiltration into the material as a means to accelerate healing.
- We have identified a variant of the mineralized collagen scaffold that contains amniotic matrix as a portion of its organic composition to improve cell activity in response to inflammatory challenge.
- We identified proteoglycans (heparin sulfate and chondroitin-6-sulfate) that can be incorporated into the mineralized collagen scaffold to maximize mineral formation in vitro.
- Defined sequestration efficiency and release kinetics for VEGF and BMP-2 in the mineralized scaffold using a mineral phase patterning approach that avoids covalent growth factor attachment.
- We showed chemical crosslinking of the mineralized collagen scaffold to increase scaffold stiffness does not affect scaffold-mediated activation of canonical BMPR signaling required for MSC osteogenic differentiation. However, stiffer crosslinked scaffolds promote MSC osteogenic differentiation via the mechanotransduction mediators YAP/TAZ and the canonical Wnt signaling pathway.
- We showed architected meshes can be integrated into collagen scaffolds to passively boost mechanical performance and actively instruct cell processes that aid osteogenicity; specifically, secretion of a factor crucial to inhibiting osteoclast-mediated bone resorption. Notably, the mechanical performance of architected Voronoi foams is predictably dependent on print parameters (porosity, strut thickness) that can be defined by generative design algorithms. Further, local changes to Voronoi mesh architecture enable localized control over composite deformation.
- We have completed in vivo trials of a composite biomaterial that combines: 1) a mineralized collagen scaffold that supports osteogenic activity; 2) a polycaprolactone mesh to aid conformal fitting in cylindrical defects; and 3) sequestered BMP-2 and VEGF to enhance regenerative healing. Six week (short term healing speed) and six month (long-term healing quality) have been completed. While bone regeneration as observed qualitatively at explant, ongoing work is completing quantitative analysis of bone healing

via immunohistochemistry (osteoprogenitor distribution; bone matrix), mechanical testing (push out strength; stiffness), and CT (ingrowth of bone into composite filled cylindrical defects), with results expected to be published in the coming year.

CONCLUSION:

Successful biomaterial implants to improve regenerative healing must meet a number of design requirements that are often in conflict with each other. They must be biocompatible, meet micro-scale mechanical needs to promote osteogenesis as well as macro-scale requirements for a mechanically robust implant, be bioresorbable, and conformal fitting within irregular defects to improve osseointegration. Through the third year of this project we have completed the design, fabrication, as well as mechanical and in vitro osteogenesis testing of a conformal fitting mineralized composite for CMF defect repair applications. We have demonstrated multiple means to incorporate and release growth factors from the scaffold. And we have shown incorporation of zinc ions into the scaffold mineral phase is a powerful stimulus to increase MSC proliferation and metabolic health. The ability to increase close contact between the biomaterial implant and the host bone as well as incorporate pro-osteogenic factors (i.e., zinc) is particularly important for improving cell recruitment and subsequent osseointegration between host and implant. We have developed alternative methods to leverage mineral-based sequestration of growth factors within the mineralized collagen scaffold without the need for chemical crosslinking. We have also defined the role of scaffold anisotropy, modification of scaffold glycosaminoglycan content, and inclusion of amnion derived matrix on guiding MSC osteogenesis. We have developed a Voronoi-based approach to create exotic polymeric reinforcement designs to address challenges of complex defect geometries (U.S. Provision Patent filed). We shut down and restarted our laboratory in response to the novel coronavirus pandemic, received formal ACURO Animal Use approval for our in vivo trials, and completed in vivo trials of craniofacial bone healing using a porcine mandibular defect model. We expect this work to generate significant enthusiasm in the regenerative medicine and biomaterials community regarding a new paradigm for orthogonally manipulating multiple design criteria to create biomaterials for large, load bearing craniofacial defects. Impact will be primarily in basic sciences associated with the design of regenerative biomaterials.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project.

(1) Lay Press:

Nothing to report

(2) Peer-Reviewed Scientific Journals:

Accepted/Published:

A.S. Tiffany, B.A.C. Harley, 'Growing pains: the need for engineered platforms to study growth plate biology,' *Adv. Healthc. Mater.*, 2200471, 2022. PMCID: PMC9547842.

In review

M.J. Dewey, R. Sun Han Chang, A.V. Nosatov, K. Janssen, S.J. Crotts, S.J. Hollister, B.A.C. Harley, 'Generative design approach to combine architected Voronoi foams with porous collagen scaffolds to create a tunable composite biomaterial,' in preparation.

(3) Invited Articles:

Nothing to report

(4) Abstracts:

Nothing to report

b. List presentations made during the last year (international, national, local societies, military meetings, etc.).

B.A.C. Harley, 'Building tissues – engineering complexity through biomaterial design,' Washington University in St. Louis, Dept. of Bioengineering, 11/2021.

B.A.C. Harley, 'Engineering porous scaffolds for regenerative medicine,' Invited speaker, Biomaterials for Regenerative Engineering session, Materials Research Society Annual Meeting, 12/2021.

B.A.C. Harley, 'Building tissues – engineering complexity through biomaterial design,' University of Oregon, Dept. of Bioengineering, 5/2022.

B.A.C. Harley, 'Building tissues – engineering complexity through biomaterial design,' University of Glasgow (Scotland), Centre for the Cellular Microenvironment, 5/2022.

B.A.C. Harley, 'Engineering complex tissues through biomaterial design,' Invited Speaker, Sex-specific differences in mechanobiology and health, World Congress of Biomechanics, Taiwan, 7/2022.

B.A.C. Harley, 'Building tissues – engineering complexity through biomaterial design,' University of Pennsylvania, Penn Center for Musculoskeletal Disorders, 9/2022.

B.A.C. Harley, 'Building tissues – engineering complexity through biomaterial design,' Harvard University, School of Engineering and Applied Sciences *Topics in Bioengineering*, 10/2022.

INVENTIONS, PATENTS AND LICENSES:

A.S. Tiffany, B.A. Harley, '*Inclusion of growth plate nodes within engineered biomaterials to augment regenerative therapy and investigate disease progression*,' Submitted to U. Illinois OTM, 2022.

M.J. Dewey, B.A. Harley, '*Composite materials and methods of making and using the same*,' U.S. Provisional Patent Application No. 63/440,325, 1/20/2023 (UIUC2020-163-01, 2022).

REPORTABLE OUTCOMES:

Mineralized collagen scaffolds fabricated with amniotic membrane matrix increase osteogenesis under inflammatory conditions. We have reported a process to incorporate amniotic derived extracellular matrix into a mineralized collagen scaffold to form a mineralized collagen–amnion composite biomaterial. We reported inclusion of amniotic membrane matrix increases scaffold mechanical properties without affecting the osteogenic capacity of porcine adipose-derived stem cells in the mineralized collagen scaffold. Incorporation of amniotic membrane matrix promotes increased osteogenic capacity in response to inflammatory media challenge. Mineralized collagen–amnion scaffold may provide a beneficial environment to aid craniomaxillofacial bone repair, especially in the course of defects presenting significant inflammatory complications.

Anisotropic mineralized collagen scaffolds accelerate osteogenic response in a glycosaminoglycan-dependent fashion. We have adapted the lyophilization process used to fabricate porous collagen scaffold, incorporating a directional solidification approach to generate anisotropic mineralized collagen scaffolds containing aligned tracks of ellipsoidal pores. Notably, while pore anisotropy did not increase short term human mesenchymal stem cell (MSC) migration, anisotropic scaffolds promoted increased bone mineral synthesis in vitro. Further, altering scaffold glycosaminoglycan content to contain heparin sulfate or chondroitin-6-sulfate further increased mineral formation in vitro. These findings show scaffold microstructural and proteoglycan modifications represent a powerful tool to improve MSC osteogenic activity.

Sequential sequestrations increase the incorporation and retention of multiple growth factors in mineralized collagen scaffolds. We have developed a process to decorate mineralized collagen scaffolds with activity inducing growth factors after scaffold fabrication. We showed sequential exposure to modified simulated body fluid treatments enable sequential sequestration of bone morphogenetic protein 2 and vascular endothelial growth factor into mineralized collagen scaffolds without additional chemical crosslinking steps. This method allows scaffolds to: 1) sequester 60–90% of growth factor from solution without additional crosslinking treatments; and 2) retain high levels of individual (>94%) or multiple (>88%) growth factors that can be layered into the material via sequential sequestration steps. Sequentially sequestering growth factors allows prolonged release in vitro (>94%) and suggests the potential to improve healing of large-scale bone injury models in vivo.

Stiffness of Nanoparticulate Mineralized Collagen Scaffolds Triggers Osteogenesis via Mechanotransduction and Canonical Wnt Signaling. We report the consequences of chemical crosslinking of mineralized collagen scaffolds on human mesenchymal stem cell (MSC) osteogenicity. Carbodiimide crosslinking increases the mechanical stiffness of the scaffolds. Mineralized collagen scaffolds activate canonical BMPR signaling pathways regardless of crosslinking status. However, stiffer crosslinked mineralized collagen scaffolds promote MSC osteogenic differentiation via the mechanotransduction mediators YAP/TAZ and the canonical Wnt signaling pathway. These findings provide important information regarding modification to scaffold properties to improve the efficiency of osteogenic differentiation in the absence of exogenous growth factors, a key requirement to aid surgical practicality.

Tough and tunable scaffold-hydrogel composite biomaterial for soft-to-hard musculoskeletal tissue interfaces. We reported a process to incorporate a hydrogel based zone adjacent to mineralized collagen scaffold to reduce localized strain concentrations that occur at the interface between biomaterials with dissimilar mechanical properties. While this work was largely focused on tendon-to-bone enthesis repair, mechanical mismatch exists

between mineralized collagen scaffolds and the defect margin of craniofacial bone defects, suggesting the potential use of adaptations to this technology to aid implant integration into complex craniomaxillofacial bone defects.

Tunable Voronoi reinforcements for mechanically stable implants to repair

craniomaxillofacial bone defects. The design of biomaterial implants to induce regenerative healing of musculoskeletal defects often requires they address multi-scale mechanical challenges. Notably, material porosity, essential for cell-infiltration and diffusive transport, and mechanics, to promote surgical handling and implant stability, are often in direct conflict. Craniomaxillofacial bone defects occur congenitally, often after high-energy impacts, and are large and irregularly shaped. We have recently described a mineralized collagen scaffold able to promote bone formation in vivo and mineralization by mesenchymal stem cell differentiation in vitro. However, while cell bioactivity and oxygen/nutrient biotransport in these porous scaffolds scale with material porosity, mechanical performance scales inversely with porosity and is insufficient for large-scale clinical adoption. We reported an innovative scaffold-mesh composite biomaterial that uses modular polymer reinforcement elements based on Voronoi structures and a scalable generative design paradigm to create a collagen-mesh composite able to conformally fit complex defect geometries. We showed Voronoi reinforced composites fit a predictive moduli equation, create biphasic composites to localize strain during loading, and create 2D and 3D composite sheets that can be rapidly shaped and which provide conformal fitting capacity. Voronoi based composite biomaterials offer unique potential as a biomaterial that can be rapidly shaped intraoperatively to conformally fit complex defects unique for individual patients while also actively accelerating bone regeneration.

In vivo regenerative trials of growth factor loaded collagen-PCL composites to assess the speed and quality of craniofacial bone regeneration.

Inclusion of a polycaprolactone frame into the mineralized collagen scaffold significantly increased biomaterials handleability during implantation into sub-critical and critical sized porcine mandible defects. A 25-mm diameter mandible defect is critically-sized. Implanted scaffolds or collagen-PCL composites remained in the defect site for 6 weeks (10mm dia. subcritical defect) and 6 months (25mm dia. critical-sized defect). All in vivo trials have been completed and we are finishing analysis of uCT, histology, and biomechanics data, with results to be reported in scientific manuscripts submitted this year.

Future reportable outcomes:

1. All experiments have been completed. We expect to publish 1 to 2 additional manuscripts in the coming year that report the results from the already completed in vivo trials.

OTHER ACHIEVEMENTS:

Degrees obtained by personnel supported by this award:

Dr. Aleczandria Tiffany. Chemical and Biomolecular Engineering (UIUC).

Ph.D.: *Mineralized collagen biomaterials for studying bone biology in vitro*, 5/2022.

Current: Post-doctoral research associate, Dept. of Chemical Engineering, University of Toronto (Mentor: Dr. Molly Shoichet). 7/2022 – current.

Fellowships awarded to personnel supported by this award:

Dr. Aleczandria Tiffany

2021 – 2022 A.T. Widiger Fellow (excellence in health or materials science research),
Dept. of Chemical and Biomolecular Engineering, University of Illinois.

Additional honors awarded to personnel supported by this award:

Dr. Marley Dewey, Ph.D.

2020; Annual Innovation Award for Outstanding Ph.D. Thesis, Chakrapani Family Trust,
Dept. of Materials Science and Engineering, University of Illinois.

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12. Tiffany AS, Harley BAC, Growing Pains: The Need for Engineered Platforms to Study Growth Plate Biology. *Advanced Healthcare Materials*:2200471, 2022.
13. Dewey MJ, Collins AJ, Tiffany A, Barnhouse VR, Lu C, Kolliopoulos V, Mutreja I, Hickok NJ, Harley BAC, Evaluation of bacterial attachment on mineralized collagen scaffolds and addition of manuka honey to increase mesenchymal stem cell osteogenesis. *Biomaterials*, 294:122015, 2023.
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19. Grier WK, Tiffany AS, Ramsey MD, Harley BAC, Incorporating β -cyclodextrin into collagen scaffolds to sequester growth factors and modulate mesenchymal stem cell activity. *Acta Biomaterialia*, 76:116-125, 2018.

APPENDICES:

Please find appended original copies of the following work:

Quad Chart:

Final Quad Chart

Peer-Reviewed Scientific Journals:

A.S. Tiffany, B.A.C. Harley, 'Growing pains: the need for engineered platforms to study growth plate biology,' *Adv. Healthc. Mater.*, 2200471, 2022. PMCID: PMC9547842.

Technology Disclosures:

A.S. Tiffany, B.A. Harley, '*Inclusion of growth plate nodes within engineered biomaterials to augment regenerative therapy and investigate disease progression*,' Submitted to U. Illinois OTM, 2022.

M.J. Dewey, B.A. Harley, '*Composite materials and methods of making and using the same*,' U.S. Provisional Patent Application No. 63/440,325, 1/20/2023 (UIUC2020-163-01, 2022).

Polycaprolactone-collagen composite biomaterials for mandible regeneration

USAMRMC 1464004

W81XWH-16-1-0566



PI: Brendan Harley

Org: University of Illinois at Urbana-Champaign

Award Amount: \$800,000

Study/Product Aim(s)

Our goal is to demonstrate that integrating a mineralized collagen scaffold within a macroporous PCL construct will generate a multi-scale composite that enhances adipose-derived MSC osteogenesis and subsequent bone regeneration. Our aims:

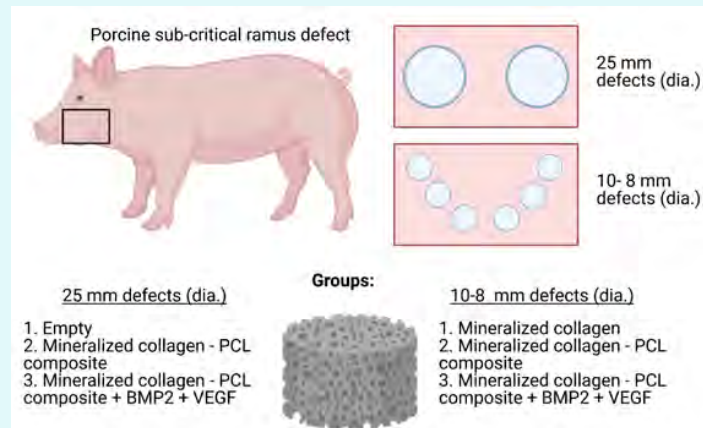
Aim 1: Validate the osteogenic potential of growth factor decorated collagen-PCL composites.

Aim 2: Examine the quality and kinetics of mandible bone regeneration using the collagen-PCL composite.

Approach

We a polycaprolactone (PCL) support cage into a mineralized collagen scaffold to form a PCL-collagen composite. We will define the improvement in adipose-derived mesenchymal stem cell osteogenesis in response to the release of growth factors (BMP-2 and VEGF) from the collagen scaffold, as well as regenerative potential of the growth factor decorated collagen-PCL composite in a porcine model of mandibular defect.

Summary of completed in vivo mandible regeneration study



Accomplishments: We completed short term (10mm dia. defect; 6 week healing) and long-term (25mm dia. defect; 6 month healing) experiments or growth factor decorated collagen scaffolds containing shape-fitting polymer reinforcement motif.

Timeline and Cost

Activities	CY	16	17	18	19
Fabrication, biophysical characterization of growth-factor decorated collagen-PCL composites					
In vitro osteogenic activity of growth-factor decorated collagen-PCL composites					
Determine kinetics of mandibular healing via growth-factor decorated collagen-PCL composites					
Define improved quality of mandibular healing using growth-factor decorated collagen-PCL composites					
Estimated Budget (\$K)		\$30k	\$200k	\$340k	\$230k

Goals/Milestones

CY16 Goal – Collagen-PCL composite fabrication

✓ Fabricate initial library of collagen-PCL composites

CY17 Goal – In vitro osteogenesis assessment

✓ Biophysical characterization of composites

✓ Define growth factor elution from collagen-PCL composites

CY19 Goal – In vivo bone regeneration assays

✓ Quantify degree of enhanced osteogenic potential of adMSCs within BMP-2 and VEGF decorated collagen-PCL composites

✓ Finalize design criteria for collagen-PCL composites for *in vivo* implantation and initiate subcritical and critical defect models

CY20/21 Goal – Quantify quality and kinetics of mandible regeneration

✓ Complete structural, biomolecular, and growth factor optimization of scaffold.

✓ Assess in vivo regeneration using a porcine mandibular defect model

Comments/Challenges/Issues/Concerns

• None

Budget Expenditure to Date

Projected Expenditure: \$800,000

Actual Expenditure: \$800,000

Updated: 20 Jan 2023



Office of Technology Management

INVENTION REPORT

CONFIDENTIAL

ABOUT THIS FORM

Thank you for reporting this invention to the Office of Technology Management. This form is the first step in a process that could lead to commercialization of this technology. Completion of the form provides the office with basic information that will help us evaluate and potentially protect and license the intellectual property.

It is not necessary to answer every question in order to submit this form.

Please also note that you can save this form and return to complete it at a later date. Once you submit this form, you will receive an email with the content of your submission. *(online version only)*

We will assign your report to a technology manager who may arrange a meeting with you. The purpose of the meeting will be to acquaint you with our process, gain a more comprehensive understanding of your technology, and define next steps.

Please note that it is the responsibility of the inventor(s) to notify the head(s) of the departments & units listed on the form below that this report has been submitted.

If you do not know an answer, if you have any questions, or would like help completing the form, please contact our office at (217) 333-7862 or otm@illinois.edu.

We look forward to working with you.

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SECTION I: CONTRIBUTORS

Please list all those who helped contribute to the conception of the ultimate working invention, using the broadest spectrum possible when identifying potential inventors. The people you include ultimately may or may not be legal inventors under U.S. patent law. Our office, assisted by outside counsel, will work to determine legal inventorship.

Please place an asterisk (*) next to the name of the inventor to whom correspondence should be sent. If any person holds a sole or joint appointment with any other university, company, or government agency, please note that fact.

*Space for additional inventors can be found at the end of this form.

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Department(s) where research was conducted: Chemical & Biomolecular Engineering			
Department(s) that administered funding, if any: Chemical and Biomolecular Engineering; Carl R. Woese Institute for Genomic Biology			

B. EXTERNAL CONTRIBUTORS

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Email	_____	Citizenship	_____
Work Address	_____		

Name	_____	Work City, State, Zip	_____
Company/Institution	_____	Home Address	_____
Phone	_____	Home City, State, Zip	_____
Email	_____	Citizenship	_____
Work Address	_____		

Name	_____	Work City, State, Zip	_____
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Email	_____	Citizenship	_____
Work Address	_____		

For the research with the above external creators, was this work done under a collaboration agreement, subaward, or sponsored research agreement negotiated by Sponsored Programs Administration (SPA)?

☐ Yes ☒ No

If yes, please provide the contract number and/or Banner number:

Sponsor Award Number	Banner Grant Code
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SECTION II: FUNDING**A. SPONSORSHIP**

Please identify all grants, contracts and other sources of funds contributing to the research that led to the invention such as Federal grants or commercially sponsored research agreements.

ADD ADDITIONAL ROWS IF NECESSARY

AGENCY/SPONSOR	SPONSOR AWARD NUMBER	BANNER GRANT CODE	Was the project description in this grant directly related to the disclosed work?
NIH	R01 DE030491		☐ Yes ☒ No
U.S. Army Medical Research and Materiel Command	W81XWH-16-1-0566		☐ Yes ☒ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

B. OTHER AGREEMENTS + INTERACTIONS

Identify any agreements or interactions that you have entered into that are related to the invention and might grant rights to a company or other party outside of the University. For example, material transfer agreements, consortia agreements, consulting agreements etc.

Did this invention use any materials that were obtained from a company or another institution?

☐Yes ☒No

If yes, please provide details:

Did you transfer any new materials related to the invention to any researcher outside of the University?

Examples of such materials include animal models, DNA, peptides, cell lines, vectors, catalysts, antibodies, small molecules and alloys.

☐ Yes ☒ No

If yes, please provide details:

Are there any contracts with third parties related to this invention, for example consulting agreements or confidentiality agreements?

☐ Yes ☒ No

If yes, please provide details:

SECTION III: INVENTION

A. INVENTION TITLE

Growth plate mimicking biomaterials

B. INVENTION OVERVIEW

i. Please provide a short summary of the invention and how it works. What problem does the invention address?

A major challenge to the tissue engineering community is need for large numbers of cells in implantable biomaterials to support tissue growth and confounding inability to rapidly generate the vasculature needed to support their long term health. It is expensive and time-consuming to generate large numbers of patient specific cells. Then the majority of these cells, without rapid vascular ingrowth, cannot be maintained in implanted biomaterial implants. We have identified a technology to replicate the structure or function of the growth plate as a means to get around this challenge.

Growth plates, or physis, are highly specialized cartilage tissues responsible for longitudinal bone growth in children and adolescents. The chondrocytes that reside in growth plates are organized into three distinct zones that are essential for proper function. We report tissue engineering strategies to replicate the structure OR function of the growth plates. Sophisticated in vitro biomaterial models of the growth plate can be used to study spatiotemporal aspects of growth plate remodeling, to investigate multicellular signaling underlying growth plate biology, and to develop platforms that address key roadblocks to in vivo musculoskeletal tissue engineering applications. We envision variations of this technology could be used to accelerate musculoskeletal tissue regeneration. Specifically, composite biomaterials that could replace the structure of the growth plate for the repair of growth plate injuries in children as well as biomaterial designs that replicate functional characteristics of the growth plate to accelerate musculoskeletal regeneration in adults. We also see opportunities to use this approach as a platform to study processes linked to disease progression (outside the body).

The invention describes strategies to incorporate hydrogel zones into porous (collagen) biomaterials. We disclose two distinct formats for developing growth plate inspired biomaterials. First, cell-seeded gradient hydrogel biomaterials that could be implanted into a growth plate, adult bone, or adult cartilage injury. The hydrogel would have distinct zones that mimic the growth plates' zones to induce integration of the biomaterial implant with the native tissue. Second, a strategy to randomly seed growth-plate inspired cell-seeded hydrogel droplets into porous biomaterial to replicate functional aspects of the native growth plate: 1) the ability to continuously generate a population of progenitor cells over a defined period of time; 2) the ability for those progenitors to migrate into the surrounding biomaterial and generate a rich population of secreted factors (growth factors, cytokines, extracellular vesicles) to promote improved healing. The former would remove the traditional requirement for incorporating large numbers of cells into a biomaterial at the time of surgical implant because the growth plate zones would endogenously produce needed progenitor cells. The latter would permit the small number of progenitor cells to produce factors that enhance healing endogenously, removing the need for injection or delivery of expensive growth factors during therapy.

ii. What are the invention's potential commercial uses?

- 1) Clinical use to repair injuries resulting from trauma, disease, or congenital issues in the growth plates, bones, and cartilage.
- 2) Research use to screen drugs for the treatment of bone diseases, specifically bone cancer.

iii. To help with our patent analysis, please describe the closest known methods, apparatus, compounds, materials, animal models, etc., as well as the disadvantages of each that are solved by your invention.

The growth plate cannot heal on its own. Surgical intervention is the primary treatment for growth plate injuries/disease, and if the damage is severe both growth plates are "closed" to prevent leg deformity due to uneven growth rates. The disadvantage of this treatment is that the growth plates are not repaired at all; rather, treatment prevents additional issues such as leg length discrepancies. Large injuries to mature bone and cartilage are difficult to repair and require implantation. A growth plate inspired material may overcome some of the challenges in healing large injuries (e.g., vascularization of the implant).

The conventional approach used to study growth plate injuries are in vivo animal models. There are animal models to study the biology of growth plates, and there is some work implanting biomaterials into animal growth plate injuries. However, these materials do not (based on our recent literature search) attempt to recapitulate the developmental process nor the spatially organized nature of the growth plate. An in vitro biomaterial model would alleviate some of these challenges.

There are current efforts to develop biomaterials to repair growth plates. These biomaterials are typically injectable or implantable. However, to the best of our understanding, none of these existing technologies actively instruct cells to mimic tissue architecture and developmental processes of the growth plate.

There are no-known biomaterials that replicate structural or functional aspects of the growth plate in regenerative medicine strategies to address musculoskeletal (bone and cartilage) tissues. Biomaterials containing developmentally-inspired structures or processes may improve regeneration in mature tissues by allowing cells to be more proliferative and repair the injury more quickly.

SECTION IV: DEVELOPMENT

A. DETAILS + SUPPORTING DATA

Please attach any published or unpublished papers, write-ups, dissertations, figures, or materials that you may have related to this invention.

ADD ADDITIONAL ROWS IF NECESSARY

PAPER/FIGURE/MATERIAL	PUBLISHED?

Growing pains: the need for in vitro models of the growth plate	☐ Yes ☒ No
	☐ Yes ☐ No
	☐ Yes ☐ No
	☐ Yes ☐ No

B. PRIOR PUBLICATIONS

To help with our patent analysis, please list any publications and patents (your own or other people's) that describe ideas closely related to your invention.

ADD ADDITIONAL ROWS IF NECESSARY

PAPER/FIGURE/MATERIAL

W. Li, R. Xu, J. Huang, X. Bao, B. Zhao, Treatment of rabbit growth plate injuries with oriented ECM scaffold and autologous BMSCs, Sci Rep 7 (2017) 44140

A. Clark, J.Z. Hilt, T.A. Milbrandt, D.A. Puleo, Treating Proximal Tibial Growth Plate Injuries Using Poly(Lactic-co-Glycolic Acid) Scaffolds, Biores Open Access 4(1) (2015) 65-74.

A.G. Erickson, T.D. Laughlin, S.M. Romereim, C.N. Sargus-Patino, A.K. Pannier, A.T. Dudley, A Tunable, Three-Dimensional In Vitro Culture Model of Growth Plate Cartilage Using Alginate Hydrogel Scaffolds, Tissue Eng Part A 24(1-2) (2018) 94-105.

C. SIGNIFICANT DATES

Please identify all past and future seminars, talks, abstracts, publications, posters, videos and web postings describing the invention. These may affect the scope of patent protection and the timing of filing.

ADD ADDITIONAL ROWS IF NECESSARY

DATE (MM/DD/YYYY)	SIGNIFICANCE OF DATE
May 9, 2022	Defense presentation in which we described the architecture of the growth plates and motivated the need for biomaterials to heal this tissue.

August, 2022	Thesis will be published which includes the review article described below (and attached). No specific description of the material is described.
2022 (unknown date)	Review article will be published about the need for biomaterial growth plate models. Paper currently in revision. No specific description of the material is described.

D. STAGE OF DEVELOPMENT

SELECT ONE:

☒ Concept / Target Identified	☐ Minimum Viable Product / Phase I Clinical Data
☐ Proof of Concept / <i>In Vitro</i> Studies	☐ Commercial Alpha/Beta / Phase II & Beyond
☐ Bench Prototype / Animal Data	☐ Commercial Launch

☐ Other

Please describe:

SECTION V: COMMERCIALIZATION**A. POTENTIAL COMMERCIALIZATION PARTNERS**

Please identify companies and individuals you think would be interested in licensing this technology.

ADD ADDITIONAL ROWS IF NECESSARY

COMPANY NAME	CONTACT NAME(S)
Allosource	

B. FUTURE USE

i. Do you think this technology could provide a foundation for the formation of a start-up company?

Unsure

ii. Do any of the inventors have interest in starting up a company to further develop, market, and support the technology?

☐ Yes. ☐ No ☒ Uncertain, I'm interested in learning more

If so, who?

SECTION VI: SIGNATURE

The corresponding inventor should sign and date:

Printed name: Alecandria Tiffany

Signature: 

Date: June 7, 2022



***A. UNIVERSITY OF ILLINOIS CONTRIBUTORS (CONTINUED)**

Name	_____	Email	_____
UIN	_____	Home Address	_____
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Name	_____	Email	_____
UIN	_____	Home Address	_____
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Phone	_____	Citizenship	_____
Name	_____	Email	_____
UIN	_____	Home Address	_____
Department/Appointment	_____	City, State, Zip	_____
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UIN	_____	Home Address	_____
Department/Appointment	_____	City, State, Zip	_____
Phone	_____	Citizenship	_____
Name	_____	Email	_____
UIN	_____	Home Address	_____
Department/Appointment	_____	City, State, Zip	_____
Phone	_____	Citizenship	_____

***B. EXTERNAL CONTRIBUTORS (CONTINUED)**

Name	_____	Work City, State, Zip	_____
Company/Institution	_____	Home Address	_____
Phone	_____	Home City, State, Zip	_____
Email	_____	Citizenship	_____
Work Address	_____		

Name	_____	Work City, State, Zip	_____
Company/Institution	_____	Home Address	_____
Phone	_____	Home City, State, Zip	_____
Email	_____	Citizenship	_____
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Name	_____	Work City, State, Zip	_____
Company/Institution	_____	Home Address	_____
Phone	_____	Home City, State, Zip	_____
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Work Address	_____		

Growing Pains: The Need for Engineered Platforms to Study Growth Plate Biology

Alecandra S. Tiffany and Brendan A.C. Harley*

Growth plates, or physis, are highly specialized cartilage tissues responsible for longitudinal bone growth in children and adolescents. Chondrocytes that reside in growth plates are organized into three distinct zones essential for proper function. Modeling key features of growth plates may provide an avenue to develop advanced tissue engineering strategies and perspectives for cartilage and bone regenerative medicine applications and a platform to study processes linked to disease progression. In this review, a brief introduction of the growth plates and their role in skeletal development is first provided. Injuries and diseases of the growth plates as well as physiological and pathological mechanisms associated with remodeling and disease progression are discussed. Growth plate biology, namely, its architecture and extracellular matrix organization, resident cell types, and growth factor signaling are then focused. Next, opportunities and challenges for developing 3D biomaterial models to study aspects of growth plate biology and disease in vitro are discussed. Finally, opportunities for increasingly sophisticated in vitro biomaterial models of the growth plate to study spatiotemporal aspects of growth plate remodeling, to investigate multicellular signaling underlying growth plate biology, and to develop platforms that address key roadblocks to in vivo musculoskeletal tissue engineering applications are described.

1. Introduction to the Growth Plates

The musculoskeletal system is an integrated system of heterogeneous tissues including muscle, tendon, ligament, and bone. The coordinated activity of these tissues is critical for structural support and movement of the human body.^[1] Interfaces between

these tissue types are typically marked by transitional zones that are essential for proper function of the skeletal system. These transitional zones often serve the purpose of connecting mechanically dissimilar tissues and facilitating movement, and often contain gradients or a hierarchically organized landscape of matrix, cells, and biomolecules. For example, the osteochondral interface is a transitional zone tissue that is composed of articular cartilage (90%), a calcified cartilage transitional tissue (5%), and subchondral bone (5%).^[2] The calcified cartilage transitional zone is a permanent ≈ 20 – $250\ \mu\text{m}$ thick layer that joins articular cartilage to subchondral bone in joints.^[3] This calcified cartilage is 10–100 times stiffer than articular cartilage but less stiff than subchondral bone when there is the same mineral content,^[4] and serves to reduce stress between mechanically dissimilar articular cartilage and bone. Another example of a transitional tissue is the enthesis, a permanent fibrocartilage tissue that joins connective tissue (e.g., tendons

and ligaments) to bone to facilitate joint motion.^[5] The enthesis is a stratified fibrocartilaginous tissue ≈ 250 – $500\ \mu\text{m}$ thick that contains gradients in cell phenotype, biochemical cues, mineral content, as well as matrix composition and alignment.^[6,7] Bone is 100 times stiffer than tendon and ligament,^[6] and the gradient within the enthesis protects it from failure by minimizing the strain concentrations between mechanically dissimilar tissues. While injuries to transitional zones linking other tissues to bone are perhaps the most considered, the growth plates (i.e., physis) are an important class of transitional zones inherent to bone itself.

Growth plates, or physis, are highly organized cartilage tissues ≈ 800 – $1000\ \mu\text{m}$ thick^[8] that lie between the epiphysis and metaphysis at the ends of most long bones.^[9,10] However, unlike the osteochondral interface and tendon-bone or ligament-bone entheses which are permanent transitional tissues, growth plates are temporary cartilage tissues involved in development and growth that disappear at skeletal maturity in humans.^[10,11] Kazemi and Williams provide an excellent comparison of the cartilage-subchondral bone interfaces in growth plates and mature cartilage.^[12] Notably, growth plates are avascular and aneural—relying on the vascular systems of the adjacent bone tissue to maintain chondrocyte proliferation^[13]—and are composed of chondrocytes embedded in an extracellular

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DOI: 10.1002/adhm.202200471

matrix.^[10] The biology of these chondrocytes will be described in further detail in the next sections. Growth plates are responsible for longitudinal bone growth during development driven by a process of chondrocyte proliferation, chondrocyte hypertrophy, and subsequent matrix deposition that occurs at the periphery of the growth plates.^[9,10] Cranial vault sutures, the fibrous tissues containing mesenchymal stem cells that unite bones in the skull during development, are the major site of bone growth during cranial development.^[14] Cranial vault sutures differ from the growth plates of the long bones in that they do not facilitate bone growth via a cartilage intermediate (i.e., endochondral ossification). Rather, cranial vault sutures facilitate bone growth via a process of intramembranous ossification where bone growth occurs directly by mesenchymal stem cell differentiation in response to extrinsic growth signals caused by the expanding brain. Growth plates in the long bones facilitate bone growth via endochondral ossification and a cartilage intermediate, and possess intrinsic growth potential (i.e., no external signal is required). Please see Opperman's comprehensive review for information on cranial vault sutures.^[14]

Bones are highly mineralized and vascularized tissues that provide mechanical support to the musculoskeletal system. There are four main categories of bone: long bones (e.g., femur, tibia), short bones (e.g., carpal, tarsal), flat bones (e.g., skull, mandible), and irregular bones (e.g., vertebrae, hyoid).^[15,16] Further, there are two primary bone microenvironments: dense, cortical bone and highly porous trabecular, or cancellous, bone.^[15,16] Bones generally consist of an outer exterior composed of cortical bone, an inner core of trabecular bone containing bone marrow,^[17] with the outer surface of cortical bone protected by the periosteum, which contains a fibrous connective tissue layer and a highly cellular cambium layer.^[15,18] Compositionally, bone is composed of inorganic calcium phosphate mineral (50–70%), organic matrix (i.e., collagen, 20–40%), water (5–10%), and lipids (<3%).^[15] Anatomically, long bones contain a central medullary cavity while the proximal and distal ends of the long bones are defined by discrete metaphyseal and epiphyseal zones. Long bones receive blood supply from arteries in the metaphysis and epiphysis, periosteal arteries, and the central nutrient artery.^[17,19] A dense capillary system within bone tissue is responsible for distributing oxygen and nutrients to bone resident cells: osteoblast, osteocytes, and osteoclasts.^[17] Osteoblasts are derived from mesenchymal stem cells, and they deposit collagen proteins that compose the bone extracellular matrix and are responsible for mineralization of these proteins.^[15,16,20–22] Osteoblasts terminally differentiate into osteocytes, bone cells that are embedded in the extracellular matrix and sense mechanical strain.^[16,20,22] Osteoclasts derive from mononuclear monocyte-macrophage precursor cells, and they are responsible for resorption of the mineralized bone matrix.^[15,16,20–22] Bone is constantly remodeled and maintained by reciprocal interactions mediated by osteoblasts and osteoclasts that are mediated through growth factor signaling. Notably, fibroblast growth factors (FGFs), transforming growth factor beta (TGFB), bone morphogenic proteins (BMPs), insulin-like growth factors (IGFs), and platelet-derived growth factor (PDGF) are all critical for bone homeostasis.^[21] Bone is also exquisitely mechanosensitive, with mechanical loading being a central factor mediating bone homeostasis and remodeling.^[15,20] For further information regarding bone homeostasis, please see

the following reviews for comprehensive discussions of bone physiology^[15,16,20–22] and vascularization.^[17,19]

2. Developmental Role of Chondrogenesis

Chondrogenesis is a key developmental process and, coupled with osteogenesis, generates the body's skeletal tissues.^[23] Chondrogenesis, the process in which mesenchymal cells differentiate into chondrocytes, is required for endochondral ossification, a mode of bone formation observed in most mammalian skeletal tissues with the exception of craniomaxillofacial bone.^[23] During endochondral ossification, mesenchymal cells undergo condensation and begin to differentiate into transient prechondrocytes (or chondroprogenitors).^[24] These chondroprogenitor cells are highly proliferative and will further differentiate into chondrocytes which begin depositing extracellular matrix proteins to form cartilage tissue.^[23,24] Chondrocytes mature and become hypertrophic chondrocytes, secreting osteogenic factors to trigger osteogenesis.^[23] Osteoprogenitors are recruited from the periphery of the cartilage tissue (i.e., perichondrium) and mature into osteoblasts.^[23] Osteoblasts then deposit bone matrix, forming a cuff of periosteum bone around the diaphysis of the cartilage tissue (i.e., bone collar).^[23] Osteoblast precursors invade the mineralized cartilage to replace the mineralized cartilage with bone matrix.^[23] A secondary ossification center forms through successive rounds of chondrocyte hypertrophy, blood vessel invasion, and osteoblast differentiation until the cartilage is replaced by bone.^[25] This secondary ossification center is where the growth plate, or physis, resides.^[25] Endochondral ossification continues to occur in the growth plates and leads to lengthening of the long bones. Thus, chondrogenesis is critical for the formation of the growth plates and is the primary process that takes place in the growth plates during skeletal development. In mice, longitudinal bone growth during fetal and neonatal periods involves the depletion of chondroprogenitors while later in life, during the formation of the secondary ossification center (i.e., the growth plates), chondroprogenitors are hypothesized to acquire the capacity for self-renewal.^[26] Chondroprogenitors start to express stem cell markers and undergo cell division simultaneously in the growth plates.^[26] Newton et al. hypothesize that a stem cell niche develops postnatally in the growth plates which provides a supply of chondrocytes over a prolonged growing period.^[26] Skeletal stem cells have been identified in the growth plates, and subpopulations of these cells can generate chondrocytes and osteocytes, but not adipocytes.^[27]

3. Growth Plate Injuries and Diseases

Growth plates can experience damage caused by injury and disease like any other tissue. Cartilage has a limited capacity for regeneration because of the absence of vasculature in its structure and its low metabolism, and therefore is unable to fully regenerate following injury or damage.^[28] The growth plates are cartilage tissues; thus, they are avascular and have limited capacity for regeneration. The growth plates are also developmental tissues, so their injury requires immediate attention to avoid developmental issues and provides unique challenges for clinical treatment. As a result, growth plates offer a unique opportunity for the field

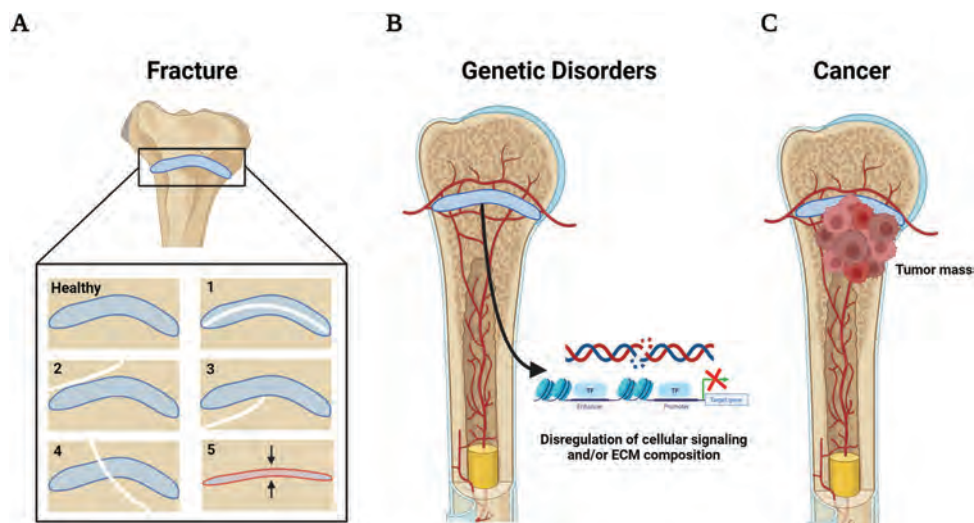


Figure 1. Injury and disease that occur at the growth plates. The growth plates, or physis, can be injured due to A) fractures which are classified into Salter-Harris fractures based on the injury location, B) genetic disorders which can alter the cellular signaling and matrix composition of the growth plates, and C) cancers which can occur at or near the growth plates and influence cell biology, matrix composition, and tissue stiffness. In each instance, the cell biology is and the extracellular matrix are altered which causes severe issues during skeletal development. Figure created with BioRender.com.

of regenerative medicine, and they may provide insight for tissue engineering strategies not just for growth plate injuries but perhaps for a wider range of musculoskeletal tissues.

3.1. Fracture and Genetic Disorders

Skeletal fractures in children are common with up to 50% of children 5–18 years old experiencing a bone fracture,^[29] and it is estimated that 15–30% of childhood bone fractures involve the growth plate.^[30] There are five types of growth plate injuries characterized by Salter and Harris^[31] (Figure 1A). Injuries occurring across the growth plates are Type I, above the growth plates are Type II, below the growth plates are Type III, through the growth plates are Type IV, and injuries that crush the growth plates are Type V^[31] (Figure 1A). The most worrisome of these injuries occurs when the injury crosses the plane of the growth plate because they interfere with the mechanics of the tissue.^[31] Growth plates do not regenerate after injury, and if left untreated these injuries can lead to angular deformity, growth arrest, and leg length discrepancies.^[32,33] Leg length discrepancy and angular deformity may lead to discomfort and altered gait, and surgical intervention is needed to prevent and treat these injuries because there is no mechanism to fully repair or regenerate the growth plates.^[32,33]

These injuries are caused when the growth plates close prematurely and lose their ability to develop and lengthen the long bones.^[33] Cartilage tissue within the growth plate will prematurely mineralize following injury, and this mineralization across the growth plate is called a “bony bridge” or “bony bar.”^[33] Research by Xian et al. suggests that bony bridge formation is caused by direct differentiation of mesenchymal cells into osteoblasts (i.e., intramembranous ossification, no cartilage intermediate, or chondroprogenitor condensation).^[34] Chung et al. demonstrated that the inflammatory response following injury

may be involved in regulating chondrogenic and osteogenic healing events, leading to bony bridge formation.^[35] Pichler et al. investigated the molecular mechanism of bony bridge formation, and their work suggests bony bridge formation begins immediately following injury.^[36] Namely, they identified pro-osteoblastic factors following growth plate injury including interleukin-6, bone morphogenic protein 2, and osteoprotegerin.^[36] The premature mineralization of the extracellular matrix and early osteogenic differentiation of mesenchymal and preosteoblast cells in the growth plate cartilage following a fracture leads to improper skeletal development; thus, there are several molecular mechanisms researchers can target when considering how to address these injuries in vivo.

Genetic disorders of the bone and skeleton can also lead to improper growth plate function and subsequent disruption of skeletal development (Figure 1B). There were 461 genetic bone or skeletal disorders classified within 42 categories in 2019.^[37] Genomic mutations were found to affect 437 different genes in 425 of the listed genetic disorders.^[37] Several genetic disorders can occur in the growth plate at the various zones; reserve, proliferative, hypertrophic; and at the edge of the growth plate; epiphysis or metaphysis.^[38,39] These disorders influence cell behavior and extracellular matrix content which subsequently disrupts growth plate function and reduces their ability to properly develop and lengthen the long bones.^[39] For example, disorders like spondyloepiphyseal dysplasia and hypochondrogenesis lead to mutations in collagen type II which is the major constituent of growth plate cartilage.^[40] Mutations in aggrecan, another major constituent of growth plate cartilage, can cause short stature and early growth arrest.^[41] Additionally, disorders such as brachydactyly type A1 and acrocapital femoral dysplasia are a result of disruptions in signaling pathways necessary for growth plate function (i.e., Indian hedgehog).^[40] Modeling growth plate biology in vitro could provide a method to study the mechanisms underlying these genetic disorders. Further, if the material

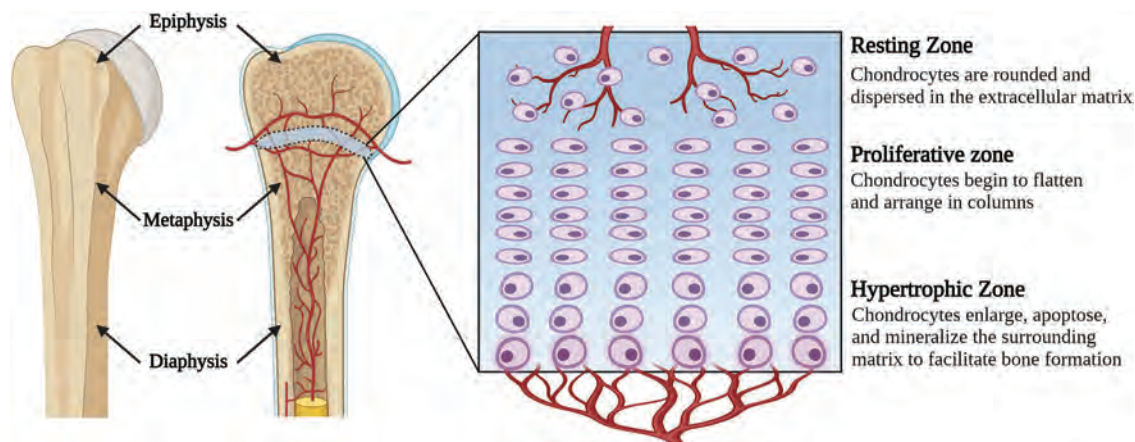


Figure 2. The architecture of the growth plates. The growth plates, or physis, are highly organized cartilage tissues located between the epiphysis and metaphysis of the long bones. They are composed of chondrocytes embedded in an extracellular matrix and contain three distinct zones: resting, proliferative, and hypertrophic. Vasculature from the epiphysis and metaphysis provide nutrients to cells in the resting and hypertrophic zones. Figure created with BioRender.com.

facilitated long-term culture of chondrocytes and the study of temporal changes to material composition, one could study how these genetic disorders progress and how they change the extracellular matrix of the growth plates.

3.2. Cancer

Osteosarcoma, while a rare form of cancer, is the most common primary malignant tumor of bone in children and adolescents.^[42] Osteosarcoma has a 5 years survival rate of $\approx 70\%$,^[43,44] but this survival rate drops to $\approx 30\%$ once metastasis has occurred.^[44] Primary treatment methods for osteosarcoma are chemotherapy and resection of affected tissues.^[45] Because osteosarcomas often occur near the growth plates in developing bones (Figure 1C), care must be taken when treating pediatric patients to prevent damaging skeletal development.^[46] Broadhead et al. have an excellent review on the molecular pathogenesis of osteosarcoma where they discuss osteosarcoma occurrence and progression.^[47] In this review, they reference studies that have shown a correlation between rapid bone growth during puberty and osteosarcoma occurrence.^[48] Further, they reference statistics stating 56% of osteosarcomas present around the knees^[49] and discuss how the growth plates of the femur (thigh bone) and tibia (shin bone) are responsible for a significant portion of height during puberty. Rapid bone growth is even listed as a risk factor for osteosarcoma by the American Cancer Society; thus, one can consider that the developmental nature of the growth plates makes them a prime spot for osteosarcoma occurrence.

4. Tissue Engineering Strategies for Growth Plate Injury

Tissue engineering approaches commonly rely on combining cells, materials, and biologics to induce a regenerative healing response in tissues. Key considerations in successful tissue engineering strategies include determining what cells to use (e.g.,

patient-derived vs allogenic) as well as how they are integrated into the biomaterial construct (e.g., seeded into the material prior to or at the time of implant vs recruitment of endogenous cells form the surrounding wound site). Often, the initial lack of a vasculature in an implanted biomaterial severely limits cell survival. While there is a critical need for tissue engineering technologies to treat growth plate injuries in adolescents, we believe there is a critical need to develop regenerative strategies for a wider class of musculoskeletal injuries that are inspired by growth plate biology. The dynamic nature of growth plate activity makes it an exciting tissue to study for spatial and temporal understanding of developing tissues. Tissue engineered growth plates may have direct applications for disease management and injury repair in children and adolescents, and for regenerative medicine strategies in adult tissues. Finally, while many mesenchymal stem cell-based tissue engineering strategies are increasingly relying on the cells as an endogenous factor of growth factors within the wound site (rather than for their multipotent differentiation capacity), there are opportunities for growth plate inspired biomaterials to act as an endogenous factory of both cells and biomolecules to aid healing.

The next section describes the biology of the growth plates: cell architecture, extracellular matrix composition, and soluble factors. Then, we discuss current methods to study growth plate biology: explant culture, cell pellet culture, and biomaterials. Finally, we discuss future perspectives for using tissue engineering strategies to develop in vitro models of the growth plates and to study dynamic changes in cell morphology and materials composition over time.

5. Architecture and Physiology of the Growth Plates

There are three distinct zones in growth plates: reserve zone, proliferative zone, and hypertrophic zone (Figure 2). The layers of chondrocytes in growth plates and their proliferation contribute to the elongation of the long bones.^[25] The layer of the growth plate closest to the epiphysis is called the resting zone (Figure 2).

Here, the ratio of extracellular matrix (the composition of the growth plate is discussed later in this review) to cell volume is high and the cells divide slowly and are nearly quiescent.^[50,51] Chondrocytes in this region are rounded, few in number, and irregularly scattered within the cartilage matrix.^[11,54,55] Notably, these chondrocytes are hypothesized to serve as progenitor or stem-like cells in growth plates.^[11,54,55] Research suggests that the resting zone cartilage is important for orienting the developing growth plates by producing a “growth plate-orienting factor” that may direct cells to align parallel to the long-axis of the bone.^[54] Additionally, vasculature originating in the epiphysis provides nutrients to chondrocytes in this zone.^[55]

The middle layer of the growth plate is called the proliferative zone (Figure 2), and it primarily functions as a site of matrix production and cellular proliferation.^[52] In the proliferative zone, chondrocytes appear flattened, begin to divide, and are arranged in columnar structures parallel to the long bones.^[9,11,50,51] Using live cell confocal imaging, Romereim et al. showed that the rearrangement of chondrocytes into columns requires cell–cell adhesion and that chondrocytes elongate to take up space, and this is indicative of cell spreading event.^[56] In contrast, the researchers did not observe cell separation, cell body contraction, or a leading edge which would have been an indication of cell migration.^[56] Thus, they assert that chondrocyte rearrangement into columnar structures is more like cell spreading than cell migration.^[56] The columnar architecture in the proliferative zone specifies the primary direction of growth in long bones.^[56] The long axis of the flattened chondrocytes is perpendicular to the long axis of the bone,^[52] and this columnar structure is maintained via cell–cell adhesion.^[56]

The layer of growth plates closest to the metaphysis is called the hypertrophic zone (Figure 2). In the hypertrophic zone, chondrocytes begin to separate from one another, become rounded, and expand in size.^[50,52,57] Chondrocytes in this zone enlarge to four times the size of chondrocytes in the proliferative zone,^[57] stop proliferating, and undergo apoptosis.^[10,50] Terminal differentiation of chondrocytes is associated with an increase in cell volume, intracellular calcium concentration,^[52] alkaline phosphatase enzyme activity, and synthesis and secretion of Collagen X.^[9,50] Hypertrophic chondrocytes direct mineralization of the surrounding matrix^[52] and attract blood vessels and chondroclasts from the metaphysis.^[58] Blood vessels from the metaphysis invade the cartilage of the hypertrophic zone, form new vascular channels via angiogenesis, and provide access for other cell types involved in bone formation.^[59,60] Differentiating osteoblasts and osteoclasts invade the hypertrophic region via the perichondrium and remodel the cartilage tissue into bone tissue.^[53,58,59,61] This remodeling leads to new bone formation underneath the growth plate and thus bone elongation.^[61] There is some evidence indicating chondrocytes trans-differentiate into osteoblasts during vascular invasion of this zone.^[62] The hypertrophic zone is the weakest and is most likely to have a cleavage plane pass through leading to growth plate injury.^[63]

The mechanical behavior of growth plate cartilage is like articular cartilage (Young’s modulus (MPa) for articular cartilage (porcine, no age provided): ≈ 2.6 ,^[64] permeability coefficient ($\times 10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}$) for articular cartilage (bovine, no age provided): ≈ 7.6 ^[65]) due to similarities in composition and structure. The growth plates are ten times more compliant in the

longitudinal direction than the radial direction,^[10] and compressive properties are nonuniform at different depths and zones of the growth plates. Sergerie et al. evaluated the mechanical properties of the whole growth plate and of each zone in newborn pig tissue samples. They report hypertrophic and proliferative zones are the least rigid, and the reserve zone is the most rigid (Young’s modulus (MPa) for hypertrophic zone: ≈ 3.3 , proliferative zone: ≈ 3.4 , reserve zone: ≈ 11.1).^[66] Additionally, they report that the hypertrophic and proliferative zones are three time more permeable than the reserve zone (permeability coefficient ($\times 10^{-15} \text{ m}^4 \text{ N}^{-1} \text{ s}^{-1}$) for hypertrophic zone: ≈ 6.0 , proliferative zone: ≈ 5.6 , and reserve zone: ≈ 1.4 zone).^[66] Other groups have utilized nanoindentation^[67] or digital image correlation^[68] to evaluate growth plate mechanics. Eckstein et al. performed nanoindentation on 9 weeks old rabbit growth plates along with Raman spectroscopy to evaluate proteoglycan content and its influence on compressive modulus.^[67] The researchers were also able to discern differences in mechanical properties for the proliferation, upper hypertrophic, and lower hypertrophic zones.^[67] Fischnich et al. utilized digital image correlation along with stress relaxation tests to evaluate 1 month old bovine growth plates.^[68] In this work, they observed growth plate mechanics varied with location and depth within the tissue,^[68] and, like Sergerie et al.^[66] they observed a lower modulus in the hypertrophic zone compared to the reserve and proliferative zone.^[68]

Mechanical loading is critical to chondrocyte biology and extracellular matrix deposition during growth plate development. Lee et al. have an excellent review discussing the mechanical stimulation of growth plate chondrocytes, and in their search of the literature it appears that compressive loading is detrimental when applied statically or dynamically; although, dynamic loading appears to be less detrimental than static loading.^[69] This is quite interesting given that researchers have shown lack of mechanical loading leads to disruption of growth plate biology. Killion et al. showed that transecting the sciatic nerve to induce limb paralysis in mice led to disruptions in column formation in the proliferative zone of the growth plates.^[70] Further, the researchers observed disorganized collagen fibers within the growth plates in limbs that did not experience mechanical loading.^[70] Lee et al. also discuss how dynamic tension has a positive impact on growth plate chondrocyte metabolism and growth^[69] which may be of critical when engineering in vitro models of the growth plates. Taken together, there is still much to be learned about mechanotransduction within the growth plates. Clinical researchers are also evaluating the effect of physical activity on the growth plates of humans, and this work further supports that mechanical loading is necessary for growth plate biology, but overexertion can lead to significant injury. Please see Mirtz et al. for a review of the literature on growth plate physiology and clinical implications.^[71]

Recapitulating the spatial organization of cells within a biomaterial will be critical for creating an in vitro model of the growth plates. Each zone within the growth plate has a unique function and disruption to any zone leads to defects in skeletal development. Considerations for how one can promote this zonal organization and evaluate cell activity within each zone should be taken when designing a biomaterial for studies of the growth plate. Additionally, it is necessary to introduce vasculature into large-scale constructs for in vivo injuries and

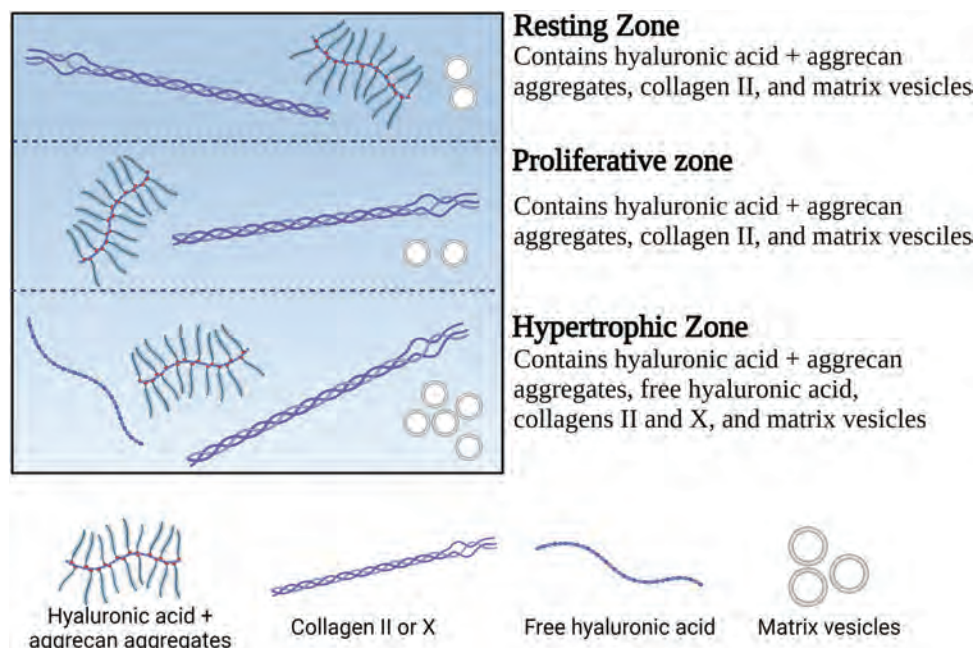


Figure 3. Extracellular matrix composition of the growth plates. The growth plates, or physis, contain multiple matrix proteins that are critical to their function. Hyaluronic acid and aggrecan aggregates along with collagen II are present in each growth plate zone. Matrix vesicles and free hyaluronic acid are most present in the hypertrophic zone, and collagen X is present exclusively in the hypertrophic zone. Figure created with BioRender.com.

understanding how angiogenesis occurs in the hypertrophic zone might improve one's ability to promote angiogenesis. Finally, we want to emphasize that this is not a trivial issue and that creating a material with this level of organization is a challenge for tissue engineering. However, the field is well equipped to tackle these challenges with the current advancements in materials fabrication techniques which will be discussed in later sections.

5.1. Extracellular Matrix Components

The primary extracellular matrix proteins in the growth plate are aggrecan, collagen, and hyaluronic acid (Figure 3). The amounts of these matrix proteins varies across the resting, proliferative, and hypertrophic regions with researchers reporting proteoglycan (i.e., aggrecan) content ranging between 36 and 67 ug per wet weight and collagen content ranging between 33 and 93 ug per wet weight.^[72] Additionally, matrix vesicles containing mineral are deposited in the hypertrophic zone and serve as nodes for mineralization and subsequent bone formation.^[73] Aggrecan, a chondroitin sulfate proteoglycan, is the primary proteoglycan in growth plate cartilage and plays a structural role in the extracellular matrix.^[74,75] Aggrecan expression is upregulated during chondrocyte differentiation^[76] and its presence is important for chondrocyte-matrix and chondrocyte-chondrocyte interactions.^[75,77,78] Aggrecan plays an important role in growth factor regulation and signaling^[79] and is essential for chondrocyte organization, morphology, and survival during limb development.^[74] The glycosaminoglycans contained within aggrecan create a negatively charged molecule that binds growth factors required for chondrocyte maturation such as In-

dian hedgehog^[80] and this negative charge enables hydration of cartilage tissue.^[75] Aggrecan forms multimolecular aggregates in cartilage, and these aggregates are composed of aggrecan monomers noncovalently bound to hyaluronic acid.^[75] Aggrecan and hyaluronic interactions are stabilized by a link protein that interacts with both molecules,^[78] with hyaluronic acid necessary for chondrocyte-matrix stabilization.^[78,81] Free hyaluronic acid, synthesized by hypertrophic chondrocytes, is present in higher concentrations in the hypertrophic zone.^[82] Free hyaluronic acid is present between chondrocytes and their immediate extracellular matrix (i.e., sulfated proteoglycans, aggrecan),^[82] though the amount of free hyaluronic acid in the pericellular space increases as chondrocytes mature.^[82]

Collagens II and X are the primary collagen types present in the growth plate. All chondrocytes produce type II collagen^[83] with the greatest expression occurring in the lower proliferative zone and upper hypertrophic zone.^[83,84] Some evidence suggests that collagen II plays a role in regulating hypertrophy in chondrocytes^[85] in addition to being a structural component of the growth plate. Collagen X is only produced by hypertrophic chondrocytes^[86] and is present in the matrix surrounding hypertrophic chondrocytes.^[83] Collagen X production is coordinated with alkaline phosphatase activity and cell size,^[87] both indicators of chondrocyte maturity. A degradation product of collagen X, the trimeric noncollagenous 1 domain, was recently discovered to be correlated with skeletal growth velocity.^[88]

Matrix vesicles in the cartilage of the growth plates were discovered in 1967.^[89] Further studies showed that these vesicles were observed in all zones within the growth plate and did not appear to be in contact with chondrocytes, and they were observed primarily in the matrix of the hypertrophic zone^[90] (Figure 3). At the time it was hypothesized that the matrix vesicles were

derived from cells and played a role in initiating calcification of the growth plates.^[90] Matrix vesicles isolated from bovine growth plates^[91] and chick growth plates^[92] displayed high levels of alkaline phosphatase.^[91,92] While some evidence suggests matrix vesicles originate from the plasma membrane of hypertrophic chondrocytes,^[73] improved understanding of the content, biogenesis, and functional activity of matrix vesicles within growth plate cartilage is still required. Ultimately, considering matrix vesicles in the design of growth plate biomaterials or in the evaluation of growth plate biomaterials is important to consider. Please see Golub's comprehensive review of growth plate matrix vesicles for the current knowledge and open questions regarding their biology and function.^[73]

Utilizing these extracellular matrix components to create growth plate-inspired materials will be important for replicating cell activity, cell arrangement, and bulk mechanical properties. Further, the expression of proteins such as free hyaluronic acid and collagen X can be used as markers to evaluate chondrocyte hypertrophy in vitro. While the resting and proliferative zones have similar composition, altering the ratio of cells to matrix could direct the spatial organization of cells in each zone. Further, since we know that cells in the proliferative zone arrange in columnar structures, one could include aligned pores or channels within the material to facilitate this arrangement. Overall, matrix composition can be used to direct cell movement and evaluate cell activity in vitro.

5.2. Regulators of Growth Plate Chondrocytes

There are several growth factors important for growth plate regulation including: bone morphogenic proteins (BMPs), cellular communication network factor 2 (CCN2), estrogen, fibroblast growth factors (FGFs), hypoxia-inducible factors (HIFs), insulin-like growth factors (IGFs), nuclear-factor κ B (NF- κ B) family, parathyroid hormone-related protein (PTHrP) and Indian hedgehog (Ihh), runt-related transcription factor 2 (RUNX2), Sox proteins, vascular endothelial growth factor (VEGF), and Wnt signaling. BMPs, FGFs, and IGFs are also critical regulators of bone homeostasis.^[21] While these factors, their functions, and relevant references are summarized in Table 1, we also include a short description of key classes of growth factors in the context of growth plate development and activity. Finally, we discuss how these factors can be used in the development of an in vitro model of the growth plate.

Bone morphogenic proteins (BMPs) are positive regulators of growth plate chondrogenesis^[93] and they are highly expressed in the perichondrium.^[94] BMPs can stimulate both proliferation and hypertrophy in the growth plates.^[95] BMPs 2, 4, 6, and 7 are expressed in all three growth plate zones.^[96] BMPs 2 and 6 are upregulated in the hypertrophic zone compared to the resting and proliferative zones.^[95] BMP signaling inhibitors are expressed in the resting and proliferative zones, and the low BMP signaling in the resting zone may help maintain these cells in a quiescent state.^[95] BMPs are more highly expressed in maturing and late stage hypertrophic chondrocytes.^[97] Differential BMP expression and signaling across the growth plate is hypothesized to be a key mechanism for the spatial regulation of chondrocyte behavior.^[95] Including BMPs that are present

in specific zones (e.g., BMP2 and BMP6 in the hypertrophic zone) may help induce cellular organization and hypertrophic morphology.

Cellular communication network factor 2 (CCN2) is strongly expressed in growth plate cartilage and is particularly expressed by pre-hypertrophic chondrocytes in the proliferative zone.^[98] CCN2 regulates cell proliferation, adhesion, migration, and differentiation of various cell types including chondrocytes.^[99,100] CCN2 binds to fibronectin^[101] and aggrecan^[102] and is required for matrix organization^[103] and enhanced aggrecan secretion.^[102,104] Studying CCN2 expression in response to varying amount of fibronectin and aggrecan in vitro may provide insight into how chondrocytes communicate with their extracellular matrix and arrange in columnar structures. CCN2-Rab14 interaction promotes the secretion of aggrecan into the extracellular space.^[105] CCN2 coordinates chondrogenesis and angiogenesis during development making it an important factor for chondrocyte maturation, cartilage remodeling, and bone formation.^[106] CCN2 stimulates the proliferation and differentiation of growth plate chondrocytes.^[107] CCN2 promotes endochondral ossification by acting on chondrocytes,^[107] osteoblasts,^[99] and osteoclasts.^[108]

Estrogen is the primary sex hormone for women, and pre-pubertal girls have eightfold higher estrogen levels than pre-pubertal boys.^[109] Estrogen positively regulates skeletal maturation and growth plate fusion,^[110,111] estrogen-resistant patients (i.e., patients with mutations in the estrogen receptor gene) grow into adulthood and lack proper growth plate fusion,^[112] and patients with high estrogen have accelerated skeletal maturation and decreased final height.^[113] Thus, estrogens play an important role in pubertal growth and growth plate closure. Estrogen receptors α and β have been identified, and researchers have shown their expression in growth plate chondrocytes of all three zones.^[114] Additionally, a membrane bound estrogen receptor (GPR30) is expressed by chondrocytes in the resting and hypertrophic zones of the growth plate.^[115] The membrane bound estrogen receptors may be a target for characterizing chondrocyte activity in an engineered growth plate. Furthermore, it is hypothesized that estrogen can stimulate growth hormone and insulin-like growth hormone expression to further stimulate longitudinal bone growth.^[111]

Fibroblast growth factors (FGFs) are positive regulators of chondrogenesis. They are expressed in all three zones, and they are involved throughout the entire process of chondrogenesis.^[116,117] FGFs' primary role is to properly pattern the developing cartilage.^[116] FGF2 interacts with members of the TGF β family of proteins.^[116] Additionally, FGF9 upregulates expression of SOX9, Ihh, Collagen II, and Collagen X.^[116] FGFs also interact with c-natriuretic peptide (CNP), and CNP counteracts the effects of FGF signaling and helps regulate extracellular matrix production and chondrocyte proliferation.^[118] Large dosage of FGF infused directly into rabbit proximal tibial growth plates accelerates vascular invasion from the metaphysis and ossification of growth plate cartilage.^[119] FGFs are involved in several processes related to growth plate biology, thus this may be an exciting target for promoting developmental processes such as angiogenesis and osteogenesis that occur in the hypertrophic zone. FGF receptor 3 (FGFR3) inhibits growth plate chondrocyte proliferation and limits the lengthening of the long bones.^[120,121] In-

Table 1. Regulators of growth plate biology.

Regulator	Action	Zone of expression	References
Bone morphogenic proteins (BMPs)	Stimulates proliferation and hypertrophy in growth plate chondrocytes.	Resting Proliferative Hypertrophic	[93–97]
Cellular communication network factor 2 (CCN2)	Regulates proliferation, adhesion, migration, and differentiation in chondrocytes. Binds to fibronectin and aggrecan.	Proliferative	[98–105, 107, 108]
Estrogen	Positively regulates growth plate fusion. Estrogen receptors α and β are expressed in growth plate chondrocytes.	Resting Proliferative Hypertrophic	[109–115]
Fibroblast growth factors (FGFs)	Positive regulators of chondrogenesis. Interacts with members of the TGF β family.	Resting Proliferative Hypertrophic	[116, 117, 119]
Hypoxia-inducible factors (HIFs)	Promote survival, maintenance, and differentiation of growth plate chondrocytes. Interacts with VEGF, a downstream target of HIF1 α .	Proliferative (center) Hypertrophic (upper)	[122–127]
Insulin-like growth factors (IGFs)	Primary regulators of longitudinal bone growth. Promotes chondrocyte proliferation and hypertrophy in chondrocytes.	Resting Proliferative Hypertrophic	[128, 129, 131–135]
Nuclear-factor κ B (NF- κ B) family	Controls normal cartilage development and pathological destruction of cartilage.	Resting Proliferative Hypertrophic	[136–139]
Parathyroid hormone-related protein (PTHrP) & Indian hedgehog (Ihh)	Regulates chondrocyte behavior and are required for bone formation. Involved in both proliferation and hypertrophy.	Resting Proliferative Hypertrophic	[50, 128, 140–145]
Runt-related transcription factor 2 (RUNX2)	Drives differentiation of proliferative chondrocytes into hypertrophic chondrocytes. Needed for osteoblast differentiation	Proliferative Hypertrophic	[52, 58, 147, 148–150]
Sox proteins	Required for the expression of chondrocyte-specific matrix proteins (e.g., Collagens II, IX, and XI). Important for chondrogenesis.	Resting Proliferative Hypertrophic	[58, 94, 126, 151, 152, 153, 155]
Vascular endothelial growth factor (VEGF)	Stimulates vasculogenesis and angiogenesis in the hypertrophic region. Critical for chondrocyte survival. Interacts with estradiol, CCN2, and HIF1 α .	Hypertrophic	[116, 127, 156, 157–161]
Wnt and PI3K/Akt signaling pathways	Necessary for the regulation of chondrocyte proliferation, hypertrophy, matrix production. and differentiation.	Resting Proliferative Hypertrophic	[162–164]

terestingly, FGFR3 plays an important role in telomerase activity suggesting telomerase sustains proliferation in chondrocytes.^[121]

Growth plates are avascular and hypoxic, thus hypoxia-inducible factors are likely critical for survival, maintenance, and differentiation of growth plate chondrocytes.^[122,123] HIF1 α is expressed in the hypoxic regions of the growth plates: the center portion of the proliferative zone and the upper portion of the hypertrophic zone.^[122] HIF1 α enhances the expression of chondrocyte marker Sox9^[124] and extracellular matrix components Collagen II^[124,125] and Aggrecan.^[116,124] HIF1 α is also required for differentiation of hypoxic prechondrogenic cells.^[126] The primary hypothesis in cartilage biology is that hypoxia modulates differentiation and condensation of mesenchymal stem cells (MSCs) into chondrocytes by activating HIF1 α .^[127] A downstream target of HIF1 α is vascular endothelial growth factor, which is discussed in a later section. HIF1 α is another excellent target for incorporation into specific zones where it is most relevant (i.e., the proliferative zone).

Insulin-like growth factors (IGFs) and growth hormone (GH) are primary regulators of longitudinal bone growth in

humans.^[128–130] These factors are expressed in all three layers of the growth plates^[131] and could be incorporated into a material at any of the desired zones. IGF1 is the major regulator of growth and normal skeletal development does not occur without proper IGF1 activity.^[132] IGF1 promotes chondrocyte proliferation and hypertrophy in the growth plates leading to bone elongation.^[133] IGF1-receptor signaling controls cell growth, survival, and differentiation in embryonic and postnatal growth plates by parathyroid hormone-related protein suppression.^[128] IGF1 and its receptor positively act on Wnt and PI3K/Akt signaling pathways,^[133] which are both important pathways in chondrogenesis^[134] and osteogenesis.^[135]

Nuclear factor- κ B (NF- κ B) signaling controls normal cartilage development^[136] and pathological destruction of cartilage.^[137] p65, a molecule in NF- κ B signaling, is expressed throughout growth plate cartilage, suggesting that the NF- κ B pathway is involved in endochondral ossification at the growth plate.^[137] IGF1 stimulates growth plate chondrogenesis by activating NF- κ B signaling.^[138] Inhibiting the NF- κ B pathway blocks growth hormone and IGF1 signaling, suppresses cell proliferation,

suppresses BMP2 expression, and ultimately leads to apoptosis of chondrocytes.^[139] Evaluating the relationship between NF- κ B and IGF1 in vitro could provide targets for materials that seek to improve osteogenesis because these factors are important for endochondral ossification.

The parathyroid hormone-related protein (PTHrP) and Indian hedgehog (Ihh) signaling pathways regulate chondrocyte behavior across the growth plate^[140] and are required for bone formation.^[141] Using numerical modeling, van Donkelaar and Huiskes showed that Ihh-related parameters mostly determined proliferation while PTHrP-related parameters predominantly determined the rate of hypertrophy.^[142] Chondrocytes proliferate and differentiate into prehypertrophic chondrocytes which express Ihh,^[143] and this Ihh interacts with cells in the perichondrium.^[143] Cells in the perichondrium then express PTHrP, and this acts on the chondrocyte PTHrP receptors to control the rate of chondrocyte differentiation.^[143,144] PTHrP receptors are found in prehypertrophic and lower proliferating zone chondrocytes.^[50,145] PTHrP and Ihh interact with IGF1 to modulate growth plate development.^[128] Mutations to this gene can cause disorders of growth plate cartilage.^[146] PTHrP and Ihh would be excellent candidates to characterize chondrocyte hypertrophy in an in vitro model.

Runx-related transcription factor 2 (RUNX2) drives proliferative chondrocytes to differentiate into hypertrophic chondrocytes.^[58] RUNX2 is expressed in the proliferative and hypertrophic zones of the growth plate, and it is needed for osteoblast differentiation and chondrocyte maturation in osteogenesis.^[147] RUNX2 expression is regulated by PTHrP^[148] and induces Ihh signaling.^[149] RUNX2 is highly expressed in perichondral cells and in osteoblasts.^[58] RUNX2 also regulates chondrocyte proliferation via the upregulation of Anthrax toxin receptor 1 (ANTXR1).^[150] Jiang et al. showed that endochondral ossification proceeds normally in ANTXR1 negative mice, but they have shorter femur and tibia bones compared to wild-type mice.^[150] The absence of ANTXR1 resulted in reduced chondrocyte proliferation,^[150] a step that is critical in bone development,^[52] and an overexpression of ANTXR1-induced chondrocyte apoptosis.^[150] Understanding the necessary balance of ANTXR1 to facilitate cartilage proliferation would benefit both in vitro models and in vivo regenerative materials.

Sox proteins are expressed at multiple stages during chondrogenesis in the growth plates and is expressed in all three zones.^[151] SOX9 is required for MSC condensation, differentiation, and the expression of chondrocyte-specific matrix proteins such as Collagen II, Collagen IX, Collagen XI, and Aggrecan.^[152] SOX5 and SOX6 also induce Collagen II and Aggrecan expression in chondrocytes and may be activated by SOX9.^[153] SOX9 is expressed in proliferating chondrocytes,^[58] acts at every stage of chondrocyte differentiation,^[58] and limits the trans-differentiation of chondrocytes into osteoblasts.^[154] In MSCs undergoing chondrogenesis, SOX9 is upregulated by BMPs.^[94] SOX9 regulates CCN2 transcription in growth plate chondrocytes^[155] and SOX9 is directly regulated by HIF1 α during chondrogenesis.^[126] SOX9 is related to multiple factors discussed in this section; thus, SOX9 would be a promising target for the development of an in vitro growth plate material. Furthermore, understanding how SOX5 and SOX6 are activated and reg-

ulated by SOX9 may be critical for mimicking chondrogenesis at the growth plates.

Vascular endothelial growth factor (VEGF) is secreted by hypertrophic chondrocytes and stimulates vasculogenesis and angiogenesis.^[116] VEGF is absent or weakly expressed in the resting and proliferative zones.^[156] VEGF expression in the lower hypertrophic region suggests it is critical for regulating vascular invasion of the growth plate,^[156,157] maintaining chondrocyte survival,^[157] and promoting osteoblast differentiation.^[158] VEGF is a target of HIF1 α ^[127] and both of these growth factor are critical for chondrocyte survival during bone growth.^[157,159] Additionally, VEGF expression in the growth plate is stimulated by estradiol^[160] and CCN2 plays an important role in VEGF localization in hypertrophic chondrocytes.^[161] Incorporating VEGF into the hypertrophic region of an in vitro biomaterial may promote angiogenesis. Further, VEGF expression could be used as a marker for hypertrophic chondrocytes.

Wnt signaling is involved in several processes related to chondrogenesis in the growth plates and occurs in all three zones.^[162] At least nine Wnts are expressed in the growth plates: Wnt 2b, 3a, 4, 5a, 5b, 7a, 7b, 9a, and 11.^[162,163] Notably, Wnt 4, 5a, 5b, 10b, and 11 are expressed relatively low in the resting zone of the growth plates.^[162] The expression of these nine Wnts is necessary for the regulation of chondrocyte proliferation and differentiation.^[163] Furthermore, Wnt signaling is necessary for endochondral ossification.^[163] β -catenin-dependent Wnt signaling is a major Wnt signaling pathway, and Wnt/ β -catenin signaling regulates multiple steps of chondrocyte function in the growth plate like proliferation, hypertrophy, and matrix production.^[163,164] Given the large number of signaling molecules related to the Wnt signaling pathways, there are multiple targets researchers can include in growth plate materials to induce or study developmental processes.

Research has shown that growth factor supplementation in biomaterial systems can improve growth plate cartilage regeneration in vivo and induce chondrogenic phenotypes in vitro.^[165] Thus, these factors can be used to influence chondrocyte biology and promote growth plate-like behavior. For example, including CCN2 in the proliferative region of the material and VEGF in the hypertrophic region of the material would mimic where these factors reside in the native tissue. Further, these factors can be used as markers for growth plate biology when studying temporal changes in vitro. For example, examining the expression of HIF1 α would provide information on the state of chondrocytes in the proliferative and hypertrophic zones. Taken together, there are several factors that are critical for growth plate biology and their functions are often related to one another; thus, researchers can utilize these factors in biomaterial systems when developing growth plate materials.

6. Studying Growth Plate Biology in 3D

6.1. Explants

Explant culture is the in vitro culture of living tissues, organs, or cells isolated from an organism (**Figure 4A**). This method retains some elements of physiologically relevant signaling. Growth plate explant cultures that contain the tissue with all three zones

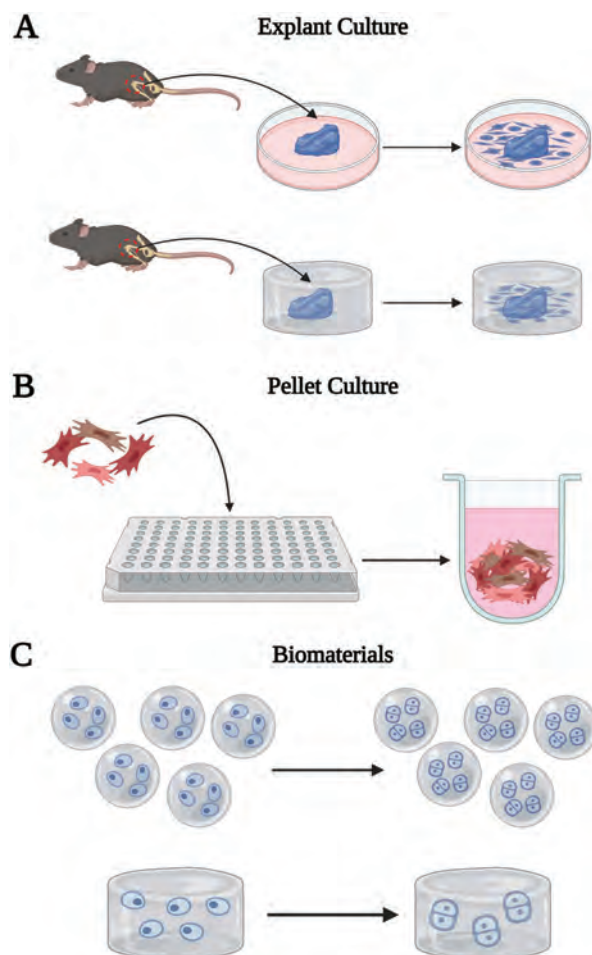


Figure 4. Methods for studying growth plate biology in 3D. A) Explant culture is the *in vitro* culture of living tissues, organs, or cells. B) Cell pellet cultures are created by centrifuging cells and then culturing the resulting pellets in media. C) Biomaterials allow the control of extracellular matrix, cellular, and growth factor composition for the *in vitro* study of biological systems. Figure created with BioRender.com.

allow chondrocytes from each zone of the growth plate to be evaluated. Explant cultures of nonhuman mammalian growth plates have been used to determine matrix protein biosynthesis^[72,166] and to study the influence of mechanical stimulation on chondrocyte behavior.^[66,72,167] While explant cultures have been useful for establishing knowledge about chondrocyte biology and the architecture of the growth plates, there are limitations to this culture system. Explants from animals are complex tissues that retain their architecture and extracellular matrix components, making it difficult to extrinsically control specific factors to investigate cell biology. However, the knowledge obtained from explant cultures can be used by tissue engineers to create biomaterial models of the growth plate that allow for more control over the culture system.

6.2. Cell Pellet Cultures

Cell pellet culture is a standard method for the chondrogenesis of MSCs, and these cultures are created by centrifuging cells

in U-shaped culture plates (or in conical tubes) and then culturing those pellets in media (Figure 4B). MSCs undergo chondrogenic differentiation in this system with serum-free media containing transforming growth factor beta (TGFB) and glucocorticoids (a class of steroid hormones).^[168] This culture system provides a 3D environment that permits direct cell–cell interaction and is commonly used for studying chondrogenesis, and cell pellet culture systems have also been used to study growth plate biology. Van Gool et al. determined that the MSCs in pellet cultures obtained a partial gene expression profile resembling growth plate cartilage.^[169] Randall et al. showed that Wnt signaling promotes the formation of growth plate columns,^[170] and Whitaker et al. showed that Smad4 is necessary for matrix synthesis by chondrocytes.^[171] Pellet cultures can also be done directly with chondrocytes.^[172] Cell pellet culture systems provide a sufficient model for chondrogenesis and growth plate formation, but they are unable to study late stages of chondrogenesis.^[169,170] Pellet culture systems allow for multiple types of analyses including histology, fluorescent microscopy, and quantitative polymerase chain reaction (qPCR). These studies also provide critical knowledge about the biology of chondrocytes and growth plate development. Disadvantages of these systems include the large number of cells needed for sufficiently sized pellets,^[170,173] necrosis in the center of pellets with time,^[173] and the lack of extracellular matrix/organization seen in native growth plates.

6.3. Biomaterials

Biomaterials provide a 3D culture environment for cells and researchers can control the composition and mechanics of these materials based on their specific needs (Figure 4C). We could only find one fully *in vitro* biomaterial-based model of the growth plate. Erickson et al. created a 3D *in vitro* model of growth plate cartilage by encapsulating primary growth plate chondrocytes in alginate beads.^[174] The chondrocytes encapsulated in these beads displayed zonal arrangement consistent with growth plate cartilage architecture.^[174] We also want to highlight the work of Wu et al. in which chondrocytes were cultured in a hydrogel for 24 h, sorted based on their extracellular matrix production, and then cultured as pellets.^[175] In this work, they demonstrated the ability to sort cells based on collagen II production, and they found that cells with and without collagen II had distinct morphology, gene expression, and histological appearance.^[175] This was a unique way to combine biomaterial systems and pellet culture systems. Biomaterial systems allow researchers to control different aspects of the culture system including material composition and stiffness, growth factor presentation, and mechanical stimulation. Biomaterials also allow the use of human cells to better mimic *in vivo* tissues. While there are few *in vitro* models of the growth plate, there have been several biomaterials designed to repair growth plates *in vivo* and we will discuss some of these advances in the upcoming sections.

7. Future Perspectives: Developing Biomaterial Tools to Investigate Disease and Injury *In Vitro*

The growth plates are highly organized tissues that are critical during skeletal development. Disease (e.g., osteosarcoma) and

injury can occur at the growth plates and surgical intervention is required to prevent deformity or growth arrest. Chondrogenesis and osteogenesis take place at the growth plates and understanding the biology of these developing tissues may provide insight for bone and cartilage tissue engineering. In vitro biomaterial models of the growth plates are needed to further our knowledge about growth plate biology. Studying the biology of chondrocyte quiescence and proliferation within the growth plates can provide insight to repair injured and diseased cartilage; further, studying osteogenesis at the growth plate may provide insight to repair critically sized bone injuries.

The growth plates are composed of three zones with distinct cell morphology and extracellular matrix properties; thus, mimicking this tissue in vitro will require materials that can recapitulate these three zones or that allows cells to self-assemble into growth plate-like structures. Biomaterial systems provide a 3D environment for cells to be cultured and studied. Biomaterials can be composed of a variety of materials (e.g., polymers, metals, minerals) and can be cultured with a variety of cell types or growth factors allowing researchers to mimic specific tissues. Advances in the fabrication of biomaterials allows researchers to create complex microenvironments to study cell behavior in the laboratory. The field of tissue engineering and regenerative medicine continues to develop exciting methods for the 3D printing of complex tissue structures, and these methods are all amenable to the development of in vitro growth plate models. Specifically, materials development strategies can be used to spatially organize cells, soluble factors, and extracellular matrix proteins that mimic the three zones of the growth plates. Further, materials evaluation strategies can be used to study temporal changes in cell morphology and material composition that one would expect to see in a model of the growth plates.

7.1. Cell and Material Patterning via 3D Printing

3D printing technologies have been popular in the tissue engineering field because they allow researchers to print complex structures using soft materials. Wu et al. printed sacrificial ink into a photopolymerizable hydrogel matrix to fabricate microvascular constructs.^[176] This was the first example of direct-write assembly—rather than layer-by-layer assembly—of complex biomimetic microvascular structures in hydrogels. However, this work required optimization of every component involved, namely, the fugitive ink, hydrogel reservoir, and filler fluid. Future work in the field would expand on this work to make omnidirectional 3D printing more accessible. Miller et al. developed a method for rapid casting of perfusable gels by encapsulating a sacrificial 3D carbohydrate glass lattices with cell-laden hydrogels composed of polyethylene glycol, fibrin, Matrigel, alginate, or agarose.^[177] This work demonstrated a method for building large and perfused tissue constructs and discussed the design space available for researchers such as matrix and cell type.

Advancements in the 3D printing of soft materials for tissue engineering have continued to push the field forward. Hinton et al. developed a method for the complex 3D printing of soft biomaterials via extrusion into a sacrificial microparticle support bath, deemed FRESH (freeform reversible embedding of suspended

hydrogels) printing.^[178] The researchers later expanded on this work and made the microparticle bath more uniform to improve the resolution of their printing.^[179] Additionally, work has been done by Hull et al. to expand the bioinks compatible with the FRESH printing method.^[180] The researchers demonstrated that a common crosslinking chemistry could be used for multiple polymers including hyaluronic acid, gelatin, and polyethylene glycol.^[180] This allows researchers to print multiple bioinks into the same sacrificial bath forming a cohesive composite material. FRESH printing, and the subsequent work done to optimize the method, provides an excellent set of tools for fabricating complex tissues in vitro. Furthermore, researchers have used microparticle baths to print cell-only ink without a biomaterial carrier. Jeon et al. directly printed stem cells into a photocurable support bath composed of alginate microgels.^[181] Once UV crosslinked, these constructs could support the printed structures and allow long-term culture in vitro. Once the printed constructs had been cultured and formed tissue constructs, they could be removed from the microgel and maintain high cell viability.

3D printing provides freedom in experimental design and biomaterial fabrication. For example, Kolesky et al. developed a multimaterial bioprinting method to create thick tissue engineering constructs containing multiple cell types and vasculature.^[182] In this work, researchers printed sacrificial ink (for vasculature) and a cell-laden bioink (containing MSCs) and cast that entire construct in another cell-laden hydrogel (containing fibroblasts) and were able to culture these large tissue constructs (>1 cm thick) for more than 6 weeks. Grigoryan et al. fabricated complex topologies in 3D hydrogels using stereolithography and food dye as photo masks.^[183] The complex and functional structures they were able to fabricate within soft hydrogels was, frankly, inspiring. The authors assert that this stereolithographic method allows for “simultaneous and orthogonal” control over tissue architecture within a biomaterial. Given the distinct zones with the growth plates, this type of control would be greatly beneficial in the development of in vitro models. Skylar-Scott et al. printed a sacrificial ink into a functional tissue composed of embryoid bodies (i.e., induced pluripotent stem cells cultures to form organoids).^[184] This method was named SWIFT (sacrificial writing into functional tissues) and allows researchers to generate organ-specific tissues with high cell density and required functionality. The printing of a sacrificial ink within the functional tissues allows perfusion (i.e., vascularization). In addition to presenting a new bio-fabrication method, the researchers were able to show that these large tissues matured and were functional in vitro when perfused for long culture periods.

3D printing allows for the direct deposition of extracellular matrix proteins, cells, and soluble factors. This level of control is amenable to growth plate materials given the highly organized nature of this tissue. Printing multiple matrices within the same construct would allow for the creation of zones with distinct features that mimic the in vivo growth plates (e.g., collagen X in the hypertrophic zone). Furthermore, the ability to print cells would allow researchers to recreate the cellular organization observed in each zone (e.g., cells arranged in columns in the proliferative zone). Finally, the incorporation of specific growth factors into each zone would promote the desired cellular response (e.g., VEGF in the hypertrophic zone to promote angiogenesis).

7.2. Alternative Cell and Material Patterning Techniques

Material fabrication can also be achieved without 3D printing. Mahadik et al. developed a microfluidic mixing platform to create 3D hydrogels with gradients of cells and matrix components.^[185] Additionally, growth factors can be covalently immobilized within these hydrogels.^[186] This biomaterial system has been used to study glioblastoma^[187] and hematopoietic stem cells.^[185,186] This fabrication method could be beneficial for growth plate models as it can create gradients of cells (chondrocytes) and extracellular components (aggrecan, collagen X) that are relevant for growth plate biology. Grolman et al. used a concentric flow microfluidic device to extrude hydrogel fibers with three distinct architectures (straight, serpentine, helically packed) and used these hydrogel fibers to study macrophage–cancer cell interactions.^[188] The researchers noticed differences in cell behavior on “straight” and “serpentine” fibers, and they hypothesize this is due to differences in cell–cell interactions between the macrophages and cancer cells on the differently shaped fibers. This work presents a unique way to utilize microfluidics to create geometries that are interesting for specific biological applications.

Research groups are also using hydrogels composed of microgels (i.e., granular hydrogels) as their tissue engineering culture system. Griffin et al. were the first researchers to publish work using poly(ethylene)glycol (PEG) microparticles covalently linked together as a 3D culture system.^[189] They named these materials microporous-annealed particle (MAP) gels. These gels can be injected and formed into any shape while providing a mechanically robust hydrogel for cell infiltration and proliferation. These gels were successful in culturing cells in vitro and in vivo. Sideris et al. then created MAP hydrogels using hyaluronic acid microparticles, explored several annealing chemistries, and further characterized these granular hydrogels.^[190] Nih et al. further showed that injection of hyaluronic acid granular hydrogels into the stroke cavity of mice increases vascularization at the stroke site and reduces the inflammatory response.^[191] More recently, Muir et al. investigated the impact of microgel fabrication techniques on the properties of granular hydrogels,^[192] and this information can be used to inform the design for one’s specific application. These granular hydrogels have been used to form cartilage tissue in vitro. Li et al. encapsulated MSCs in gelatin microgels and used these cell-laden microgels to form a granular hydrogel for cartilage formation.^[193] The researchers used NHS chemistry to assemble the microgels and the granular hydrogel, and these hydrogels were able to maintain chondrogenic differentiation better than bulk hydrogels and pellet culture. Additionally, researchers are using these microgels to increase cartilage repair in vivo. Erickson et al. utilized chitosan-genipin microgels to increase growth plate cartilage regeneration in a 28 day rat growth plate model.^[194] The properties of granular hydrogels can be tailored to one’s specific needs by changing the polymer used to form the microgels, utilizing different microparticle fabrication methods, and culturing tissue-specific cell types.

Scaffold-hydrogel composites provide another exciting avenue for exploring tissue biology. Sun Han Chang et al. fabricated a continuous triphasic scaffold with three distinct regions for bone, cartilage, and tendon.^[195] The researchers used a freeze-drying

process and a custom mold to generate a continuous triphasic scaffold for tendon-bone repair in which a compliant PEG hydrogel was incorporated between mechanically dissimilar tendinous and osseous materials.^[195] The researchers illustrated a reduction in strain at the biomaterial interface between these two dissimilar biomaterials by tuning hydrogel fabrication parameters.^[195] The design parameters discussed in this article may prove useful to other researchers developing materials for interfacial zones in the musculoskeletal system. Further, fabrication techniques that combine tissue engineering constructs for multiple tissue types may be useful for the growth plates given that they are sandwiched between bones.

Utilizing these alternative cell and material patterning techniques would allow researchers without access to 3D printing to create this transitional zone in vitro. Additionally, combining multiple aspects from these works could lead to novel composite materials with unique cell–cell and cell–material interactions to study. For example, granular hydrogels are well suited for the inclusion of a bone–cartilage interface. One could physically place chondrocyte-laden microgels near osteoblast-laden or mineralized microgels to create an interface that might lead to chondrocyte organization. Furthermore, scaffold-hydrogel composite systems are exciting candidates for growth plate biomaterials because they inherently contain an interfacial zone between the two distinct materials.

7.3. Evaluating Temporal Changes in the Extracellular Matrix

There have also been advances in how researchers can evaluate cells and their extracellular matrix in 3D biomaterial systems. Loebel et al. evaluated the deposition and remodeling of nascent protein by individual MSCs in hydrogels.^[196] This was achieved by labeling methionine analogs that would be incorporated into proteins as they are being synthesized, conjugating a fluorophore to these methionine analogs via cycloaddition, and visualizing them using confocal imaging. The researchers found that nascent proteins define the pericellular environment in hydrogel systems by masking or supplementing cues provided by the biomaterial matrix. Loebel et al. further utilized this method to examine the accumulation of matrix at the interface of chondrocytes and their hydrogel matrix.^[197] Here, the researchers reported that the deposition of nascent protein physically displaces the hydrogel around the cell, and this suggests that secreted matrix proteins mask the presentation of the biomaterial. Understanding how cells are changing their pericellular environment would be critical for the growth plates, because they are dynamic tissues where chondrocytes progressively differentiate as the tissue develops.

Further expanding on this nascent protein labeling technique, Salzlechner et al. combined the fluorescent labeling of nascent proteins with Raman spectroscopy to look at the biochemical content of cells in addition to exploring the content of the pericellular environment.^[198] Raman spectroscopy is a nondestructive, noninvasive spectroscopic technique to obtain detailed information about cells and their biochemical properties. Raman spectroscopy has been used to evaluate chondrocyte differentiation and the extracellular matrix proteins present in cartilage,^[67,199] to determine the strength and mineral quality of bone,^[200] and to

grade chondrogenic and osteosarcoma tumors.^[201] This spectroscopic technique would be beneficial in the development of in vitro growth plate models because it would allow researchers to characterize chondrocytes in each zone of the growth plate.

8. Future Perspectives: Regenerating Diseased and Injured Cartilage In Vivo

There are very few in vitro biomaterial models of the growth plate, and we believe the creation of growth plate biomaterials will be critical to understand growth plate biology and to advance cartilage and bone tissue engineering. In this section, we discuss biomaterial systems that are used to repair growth plate cartilage and mature cartilage; however, these materials are often unable to restore the developmental processes that occur in the native growth plates which is the next step for these regenerative materials.

Biomaterial systems are being developed to repair growth plate cartilage in vivo, but they are often unable to restore the developmental capabilities of the growth plate and are unable to prevent bony bridge formation and angular deformity. Alsberg et al. developed a hydrogel system containing an RGD-containing peptide sequence that promoted cell multiplication that was able to increase in mass and size when transplanted with chondrocytes into a mouse model,^[202] and this ability to change size temporally would benefit a growth plate implant. Additionally, the researchers observed bone tissue growth when chondrocyte and osteoblast-laden hydrogels were injected into the backs of mice, and this bone tissue had structures histologically similar to the growth plates and its distinct zones.^[202] Li et al. fabricated an oriented scaffold derived from rabbit growth plate cartilage and were able to efficiently prevent bony bridge formation in acutely injured rabbits; however, the researchers were unable to prevent angular and shortening deformities of the bone.^[203] The researchers used rabbit growth plate matrix microfilaments to fabricate their scaffolds and rabbit MSCs for their in vitro and in vivo studies.^[203] Clark et al. used poly(lactic-co-glycolic acid) scaffolds loaded with growth factors and cells in a rabbit model, and the researchers saw increased chondrocyte population and reduced loss of remaining native growth plate compared to current clinical treatment (fat graft).^[204] Although this work showed increased cartilage formation and reduced bony bridge formation in vivo, there was no reduction in angular deformation.^[204] Many groups use cell-laden biomaterial systems for in vivo growth plate injury repair, and for a comprehensive review of regenerative medicine approaches for growth plate repair, please see Shaw et al.^[205] In this review, the authors describe regenerative medicine strategies for pediatric growth plate injuries and their associated limitations.^[205] They concluded that cell-laden biomaterials produced better results than empty biomaterials and that cells are critical for restoring growth plate cartilage; thus, biomaterials should be designed to instruct cellular behavior to recapitulate the native growth plates.^[205]

In addition to biomaterial solutions for growth plate repair (i.e., developing, young cartilage), researchers are developing biomaterial solutions to repair mature, adult cartilage in vivo. These materials have applications for both injured cartilage (i.e., trauma) and diseased cartilage (i.e., osteoarthritis). Mata et al. used alginate hydrogels seeded with human dental pulp stem

cells or primary rabbit chondrocytes to regenerate cartilage in a rabbit model.^[206] The researchers saw improvement in cartilage regeneration with cell-seeded hydrogels to compare to unseeded controls, and they observed improved tissue regeneration when human dental pulp stem cells were used compared to primary chondrocytes.^[206] Apelgren et al. developed a bacterial nanocellulose bioink and seeded these materials with human chondrocytes for cartilage regeneration.^[207] The researchers observed chondrocyte proliferation and tissue integration after 60 days in a mouse model, and the tissues had no signs of necrosis.^[207] Many groups use cell-seeded biomaterials for mature cartilage repair in vivo, and for a thorough review of biomaterials for cartilage repair, please see Ansari and Eshghannmalek^[208] and Theodoridis et al.^[209] Ansari and Eshghannmalek discuss a variety of biomaterials used for cartilage tissue engineering including scaffolds, bio-adhesives, and nanomaterials.^[208] The biomaterials discussed in this review include those composed of natural materials (e.g., alginate) and synthetic materials (e.g., polycaprolactone).^[208] Theodoridis et al. focus specifically on cell-laden biomaterials composed of natural materials that have been used to regenerate cartilage tissue in vivo.^[209] The authors discuss preclinical and clinical trials in detail for a variety of biomaterials composed of materials including hyaluronic acid, alginate, silk, and collagen.^[209]

We believe expanding these works to include developmentally inspired design (i.e., cell organization that resembles the growth plates, soluble factors necessary for chondrogenesis, matrix vesicles as seen in the hypertrophic zone) will improve their regenerative outcomes. Furthermore, studying the underlying mechanisms that lead to chondrogenesis, osteogenesis, and angiogenesis at the growth plates during skeletal development can lead to novel design principles researchers can use to improve regeneration in mature tissues.

9. Future Perspectives: Opportunities for Innovative Bone Tissue Engineering Solutions

Understanding and leveraging growth plate biology may be crucial in bone tissue engineering and several groups are working toward this goal. We already mentioned the work of Alsberg et al., in which osteoblast-laden hydrogels were able to grow in size and developed into bone tissue with structures histologically like growth plates.^[202] Cunniffe et al. fabricated scaffolds derived from growth plate extracellular matrix for repair of large bone injuries.^[210] The researchers observed that scaffolds derived from the extracellular matrix of growth plate tissue supported osteogenesis of MSCs in vitro, and they observed accelerated mineralization and vessel in-growth in an in vivo rat model compared to untreated injuries.^[210] In this work, decellularized growth plate tissue was homogenized into a liquid slurry and freeze dried to create the growth plate-derived materials.^[210] Hall et al. used human periosteum-derived cells to produce microspheroids that are differentiated into "callus organoids" and these organoids have the capacity to form ectopic bone when implanted into mice.^[211] The researchers observed that these organoids assemble over 24 h in vitro into large, engineered tissues with the ability to heal critically sized injuries in the long bones of mice.^[211] They also obtained temporal gene expression during organoid formation that mirrored the endochondral ossification process,^[211] a process

which is occurring in the growth plates during skeletal growth. Hung et al. and Whitehead et al. have used spheroids laden with MSCs for bone tissue engineering with promising results.^[212] Further, Vorwald et al. have shown success with neovascularization using endothelial cell-laden spheroids.^[213] Vascularization is critical in bone tissue engineering because bone is highly vascularized, and vascularization is critical for bone development because vessels infiltrate the hypertrophic zone of the growth plates to facilitate bone growth. Combining the design principles and methods from these different researchers could lead to exciting advancements for bone tissue engineering. These works and their design principles inspire our group to think about how the biology of developing tissues might aid in the engineering of mature bone tissue.

9.1. Creating “Osteoprogenitor Factories” using Growth Plate-Inspired Biomaterials

We believe the growth plates can be viewed as factories of osteoprogenitors because the growth plates are responsible for the lengthening of the long bones and serve as a site for osteogenesis during skeletal development. By understanding growth plate biology, one can help the treatment and regeneration of large-scale bone injuries. Lenas et al. discussed growth plates in their review article on “developmental engineering” in which they discuss the growth plate as an example of “tissue variable interdependency” due to the growth plates highly organized architecture in terms of cell differentiation state, extracellular matrix components, and tissue organization.^[214] More recently, Sheehy et al. discuss a shift toward developmentally inspired strategies for bone tissue engineering.^[215] In this review, they discuss using biomaterial systems to induce endochondral ossification (bone development through a cartilage intermediate) to circumvent common challenges in bone tissue engineering such as vascularization.^[215] We believe this can be achieved by combining aspects from multiple research efforts. For example, one could imagine creating hydrogel spheroids derived from decellularized growth plate extracellular matrix (inspired by the works of Li et al.^[203] and Cuniffe et al.^[210]) and encapsulating chondrocytes and endothelial cells (inspired by the works of Vorwald et al.^[213]) to promote a process that resembles endochondral ossification. There is an abundance of biomaterials design and fabrication techniques that are perfectly amenable to growth plate tissue engineering.

10. Conclusion

The growth plates, or physis, are highly organized cartilage tissues responsible for longitudinal bone growth in children and adolescents, and these tissues provide an exciting motivation for regenerative medicine strategies. The growth plates are organized into three distinct zones with each zone containing chondrocytes that present unique morphology and function. At skeletal maturity, the growth plates close and are replaced by bone. Disease or injury to any of the three growth plate zones can lead to growth abnormalities. There are no biomaterial solutions to repair growth plate injuries, and the most common treatment is mechanical fixation to minimize leg length discrepancies and

angular deformities. Therefore, there is a clinical need for tissue engineering solutions for growth plate injuries in children and adolescents. Growth plate biology can also be used to inform the design of biomaterial systems. We conclude that there is an important opportunity for our community to leverage mechanisms of growth plates (i.e., chondrogenesis, osteogenesis, neovascularization) to improve repair in mature tissues of the musculoskeletal system such as cartilage and bone. We propose extending advancements in tissue engineering strategies and developmentally inspired design, and we believe there are exciting opportunities to leverage traditional biomaterials strategies (e.g., decellularized matrices, freeze drying), additive manufacturing strategies (e.g., FRESH printing, SWIFT printing), and organoid technologies to create a new generation of developmentally inspired regenerative medicine materials. Specifically, we believe recapitulating the cell and matrix organization along with supplying soluble factors relevant to each zone will lead to the development of an in vitro model of the growth plates.

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Conflict of Interest

The authors declare no conflict of interest.

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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

TITLE: COMPOSITE MATERIALS AND METHODS OF MAKING AND USING THE SAME

Inventor(s)/Applicant(s):

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Submitted herewith are:

- ☒ 30 pages of specification, 4 pages of claims, and 1 page of an Abstract
- ☒ 14 sheet of drawings
- ☒ Application Data Sheet

FEE CALCULATION											
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Basic Filing Fee											\$120.00
Total no. of pages	49	Multiplied by 75% for EFS page discount	=	37	-	No. pages in basic fee	100	=	No. of extra pages, divided by 50, rounded up to nearest whole number	0	x \$210.00 = \$0.00
TOTAL FILING FEE											\$120.00

- ☒ Small entity status is claimed for this application.
- ☒ \$120.00 is being submitted herewith via EFS to cover the above-listed fee.
- ☒ The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government. The name of the U.S. Government agency and the Government contract number are: Grant No. W81XWH-16-1-0566 awarded by the United States Army/Army Medical Research and Material Command, and Grant Nos. R01 DE030491 and R21 DE026582 awarded by the National Institutes of Health.

- ☒ The Director is hereby authorized to charge any additional fees that may be required in connection with this filing, or credit over-payment, to Deposit Account No. 02-4550.
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COMPOSITE MATERIALS AND METHODS OF MAKING AND USING THE SAME

FIELD

- 5 This disclosure relates to composite materials and methods of their use, for example in methods of repairing bone defects.

ACKNOWLEDGMENT OF GOVERNMENT SUPPORT

- 10 This invention was made with government support under Grant No. W81XWH-16-1-0566 awarded by the United States Army/Army Medical Research and Material Command, and Grant Nos. R01 DE030491 and R21 DE026582 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

- 15 Creating biomaterials to repair missing segments of bone is challenging not only due to the variety of cell types involved in bone regeneration, but also the complex mechanical properties. Craniomaxillofacial (CMF) defects are especially difficult to repair due to the substantial amount of bone missing, which will not regenerate without surgical intervention. Examples of these include cleft palate birth defects, loss of bone
20 volume from dentures and cancer resection, and battlefield injuries. Many of these defects are irregular in size and shape, especially if these result from birth defects and trauma injuries. Thus, the repair of these defects not only requires a material able to promote new bone formation, but one that can be designed for any size and shape. Additionally, these must be stiff enough to promote new bone formation, but also fit
25 well to the defect site during implantation and be easy for the surgeon to handle. This creates the need for very precise design of the mechanical properties of biomaterials.

SUMMARY

Disclosed herein are composite materials with properties that make them particularly useful for repair of irregular bone defects, such as CMF defects. Also provided are methods of making and using the disclosed materials.

5 In some embodiments, a composite material including a macroporous structure having a Voronoi architecture and a microporous biomaterial integrated into the macroporous structure are provided. Embodiments of the composite material include a macroporous structure that is a mesh including a plurality of pores. In some examples, the macroporous structure includes fibers connecting points defining the Voronoi
10 architecture. In embodiments, the composite material includes a macroporous structure with at least a first portion and a second portion, and wherein the pore size of the first portion is greater than the pore size of the second portion. The macroporous structure may be two-dimensional or three-dimensional.

In some embodiments, the biomaterial included in the composite material
15 includes collagen, glycosaminoglycans, calcium phosphate, or a combination of two or more thereof. In one example, the biomaterial is mineralized collagen.

Also provided are methods of making the composite material. In some examples, the method includes contacting a macroporous structure having a Voronoi architecture with a solution including a liquid suspension of a biomaterial and removing
20 the liquid from the solution, for example by lyophilization. The methods may also further include preparing and/or designing the macroporous structure. In some examples, the macroporous structure is produced using an additive manufacturing method.

In some embodiments, the disclosed composite materials are used to treat a bone
25 defect in a subject. The methods include implanting the composite material in the bone defect. In some examples, the bone defect is a craniomaxillofacial bone defect. In some examples, the methods also include shaping the composite material to the bone defect of the subject prior to implanting the composite material.

The foregoing and other features of the disclosure will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

5

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a schematic of an exemplary macroporous structure embodiment.

FIG. 1B is a schematic of an exemplary biphasic macroporous structure embodiment.

FIGS. 2A and 2B are schematics depicting exemplary Voronoi structures (FIG. 2A) and exemplary objectives of the current disclosure (FIG. 2B).

FIGS. 3A and 3B show relationship of changes in porosity of small Voronoi cubes to a predictive modulus equation. FIG. 3A is a schematic showing four Voronoi cube designs (10 mm side length) printed with photopolymerizable white resin and their design labels. FIG. 3B shows data from mechanical compression of Voronoi cubes to yield a Young's Modulus. Predictive modulus was compared to experimental modulus of each resin to demonstrate validity of the predictive modulus equation. X-axis denotes design labels in FIG. 3A.

FIG. 4 is a series of graphs showing representative compressive loading and unloading curves for laser-sintered PCL Voronoi cubes (25 mm side length). The bottom right graph is a representative stress-strain curve of laser-sintered PCL Voronoi cubes of different thicknesses overlaid on the same graph.

FIGS. 5A and 5B show the effect of strut thickness on mechanical behavior of Voronoi structures. FIG. 5A shows Voronoi cube structures (25 mm side length) made of laser-sintered PCL compared to their .stl design. FIG. 5B shows Voronoi cubes that were compressively loaded and unloaded to determine Young's Modulus and percent deformation after loading. The normalized Young's Modulus of the predictive equation was compared to the normalized Young's Modulus of experimental data. ** indicates the 1.5 mm group is significantly ($p < 0.05$) greater than the other two groups. * indicates the 1.0 mm group is significantly ($p < 0.05$) greater than the 0.7 mm group. Data represented as average $\pm$ standard deviation.

FIGS. 6A-6C illustrates the structure of mineralized collagen scaffolds reinforced with a biphasic Voronoi 3D-print. FIG. 6A is a 3D rendering of biphasic Voronoi design, including a denser region and a porous region. FIG. 6B shows the biphasic Voronoi 3D-print made of white photopolymerizable resin (left), mineralized collagen scaffolds (center), and a mineralized collagen scaffold reinforced with the biphasic Voronoi 3D-print (right). FIG. 6C shows SEM images of mineralized collagen infill into biphasic Voronoi 3D-prints. 1) represents the dense 3D-print region, in which there are areas where the collagen was unable to infiltrate. 2) represents the porous 3D-print region, in which it is visible that porous mineralized collagen surrounds the 3D-print and both are well-integrated.

FIGS. 7A and 7B show analysis of isotropy in Voronoi structures of various thicknesses. FIG. 7A schematically illustrates Voronoi cube structures with 0.7 mm strut diameter (25 mm side length) made of laser-sintered PCL are compressively loaded and unloaded on each of the three axis represented as side 1-3. FIG. 7B shows Young's modulus and percent deformation compared between the three compressed axes. A representative stress-strain curve of the three groups tested demonstrates loading behavior. * indicates one group is significantly ($p < 0.05$) greater than another indicated group. Data are represented as average $\pm$ standard deviation.

FIGS. 8A and 8B show mechanics of mineralized collagen scaffolds reinforced with a biphasic Voronoi 3D-print. FIG. 8A is a representative stress-strain curve of Voronoi biphasic 3D-prints under compression. Numbers on the curve represent images of the compressed print on the right. The curve is broken into two segments, an area of strain density on the "porous" region of the 3D-print and an area of strain density on the "dense" region of the 3D-print. FIG. 8B shows stress-strain curves of the porous and dense regions of the biphasic 3D-print, demonstrating the elastic regimes, collapsed plateaus, densification regimes, and for the porous 3D-print, catastrophic failure. 3D-prints measured 20 mm x 6 mm x 6 mm (length x height x width).

FIGS. 9A-9D show additional mechanics of mineralized collagen scaffolds reinforced with a biphasic Voronoi 3D-print. FIG. 9A is a graph of Young's Modulus of mineralized collagen scaffold, the porous and dense region of the biphasic Voronoi

3D-print, the porous and dense region of the mineralized collagen scaffold reinforced with a biphasic Voronoi 3D-print. All groups are significantly different ($p < 0.05$) from each other. Data are expressed as average $\pm$ standard deviation ($n=8$). FIG. 9B shows a representative stress-strain curve of biphasic Voronoi 3D-prints. FIG. 9C is a graph of predictive and experimental modulus (E^*) of rectangular Voronoi 3D-Prints. The normalized Young's modulus of the porous and dense region of the biphasic print and the Young's modulus of a uniform rectangular Voronoi print were all compared to the predictive normalized moduli based on cube Voronoi structures printed with the same material (see FIG. 6A) ($n=8$). FIG. 9D is a representative stress-strain curve of mineralized collagen reinforced with biphasic Voronoi 3D-print.

FIG. 10 illustrates stress-strain profiles of uniform and biphasic Voronoi-mineralized collagen composites. Biphasic and uniform Voronoi composites were compressed and images during compression were analyzed for localized strain ($n=6$, representative images). Images of the composites are displayed with increasing compression and matching strain profiles in the direction of compression (y axis). Red indicates low strain and blue indicated regions of high strain. Biphasic Voronoi structures are oriented with the more porous region in contact with the compression platen (top of profile and images). Composites measured 20 mm x 6 mm x 6 mm (length x height x width).

FIG. 11 shows line scans of the strain on biphasic Voronoi composite and uniform Voronoi composite from FIG. 10 were created from Digital Image Correlation data. Average strain at a global applied 4.4% strain was plotted as a function of position for each sample, with the average (black line) and standard deviation (interval contained by gray lines) of strain magnitude along the entire length of each scaffold. The porous region is demonstrated by a grey background and the dense region with green ($n=6$).

FIGS. 12A and 12B illustrate shape-fitting ability and fabrication of 2D and 3D Voronoi-mineralized collagen composite sheets. FIG. 12A is images of 2D and 3D Voronoi composites and 3D-prints. The Voronoi 3D-print row includes images of PCL 3D-printed sheets. Scale bar represents 15 mm. The Voronoi composite row includes

images of the PCL 3D-print combined with mineralized collagen to create a composite material. Scale bar represents 15 mm. The composite integration row includes representative SEM images of the PCL 3D-print within the mineralized collagen scaffolds demonstrating integration of the 3D-print with surrounding collagen. Images are false colored blue to represent PCL 3D-print and red/yellow to represent mineralized collagen. SEM scale bar represents 250 μm . FIG. 12B shows push-out testing of mineralized collagen scaffolds, 2D Voronoi-collagen composites, and 3D Voronoi-collagen composites. Push-out testing was performed on two cylindrical defect sizes (11.5 mm diameter and 10.8 mm diameter). There were no significant ($p < 0.05$) differences in the pushout force required by the 2D Voronoi composite and the mineralized collagen scaffold. * indicates significant difference between the 3D Voronoi composite and the mineralized collagen scaffold at the respective defect size. ^ indicates significant difference between the 3D Voronoi composite between the two defect sizes. The last figure includes a representative load vs. extension curve for the composites and mineralized collagen scaffolds in the 11.5 mm defect size. Data are represented as average $\pm$ standard deviation ($n=8$).

FIG. 13 is additional SEM images demonstrating integration of PCL 3D-print and mineralized collagen in 2D (left) and 3D (right) Voronoi-mineralized collagen composites. Scale bar represents 250 μm .

FIGS. 14A-14D is a schematic showing an exemplary overview of an embodiment. FIG. 14A shows creating a Voronoi structure within any space. First, a set of “seed” points are added to the space. Then phantom lines (green) are drawn between these points, with phantom points (green) in the middle of these areas. Finally, the lines of the Voronoi structure (blue) are drawn between the phantom lines connecting the phantom points. FIG. 14B shows representative stress-strain curve of foams, such as Voronoi structures. These structures are characterized by a linear elastic regime, a collapsed plateau, and densification. Increasing the density of Voronoi structures increases the modulus (elastic regime). FIG. 14C shows that Voronoi structures can be added to any size or shape design and can achieve conformal fitting by the reversible collapse of pores. Additionally, the thickness of the individual struts in

the architecture and the material it is comprised of can lead to tunable strength and elasticity. Finally, strain can easily be localized by printing these designs with regions of variable porosity or density of material. FIG. 14D illustrates an exemplary use of incorporating tunable Voronoi 3D-printed architectures into mineralized collagen scaffolds for use in bone repair. Mineralized collagen scaffolds offer excellent bioactivity and osteogenic properties due to the mineral, glycosaminoglycans, collagen, and porous nature of this material, however, this porosity also lends to these being very soft structures and more difficult to handle in bone repair situations. 3D-printed Voronoi structures offer a mechanically stable material with tunable mechanics, however, the macro-scale porosity and print material may not be as osteogenic as mineralized collagen scaffolds. Combined together, mineralized collagen and 3D-printed Voronoi structures have the ability to promote osteogenesis while also maintaining strength. Scale bar represents 100 μm .

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DETAILED DESCRIPTION

One method to address mechanical fitting problems is through “shape-fitting” designs. Shape-fitting can be achieved through mechanical or chemical means in materials. Chemical modifications of materials have been used in many instances for shape-fitting, such as a biomaterial which can be heated outside the body to allow for surgical shaping to the defect space, and then cooled to body temperature to enable hardening and fit of the implant within the defect. These have been shown to regenerate bone, however, heat treatment of implants may only be possible for specific biomaterial chemical compositions (such as polymers) and may even exclude bio-derived polymers such as collagen, which denature at high temperatures. Alternatively, mechanical methodologies implement design of the structure of implants to be contracted with force by the surgeon, placed within a defect space, and released to fit against the defect. Another strategy has included micro-CT scanning of the patient’s defect and 3D-printing implants with uniform pores to fit within this missing space.

Mineralized collagen scaffolds are porous biomaterials comprised of collagen, mineral, and glycosaminoglycans, and have been shown to induce osteogenesis and

bone repair without supplemental growth factors or osteogenic media. Although these scaffolds promote bone formation, they are extremely soft with a Young's Modulus on the order of hundreds of kilopascals when dry and tens of kilopascals when hydrated and crosslinked. In comparison, normal human cortical and cancellous bone have

5 Young's Moduli on the order of one to tens of gigapascals. Thus, there remains a need to reinforce these soft scaffolds to promote bone formation while being mechanically robust and flexible to fit well to defect sites.

Provided herein are designs utilizing Voronoi architectures. Voronoi architectures are random, open-porous structures generatable into any size and space by

10 a set of random "seed" points, which make up the center of the pores, or cells, of the Voronoi design (FIG. 2A). The addition of Voronoi structures as tunable reinforcements to soft mineralized collagen scaffolds is disclosed herein. As described below, Voronoi structures with well-defined mechanics based on a predictive equation can be created (for example by 3-D printing), and then implemented within mineralized

15 collagen scaffolds to create a shape-fitting and bone regenerative implant.

I. Composite Materials Utilizing Voronoi Architectures

Provided herein are composite materials that in some examples are suitable for use as implants in repair of bone defects, such as CMF defects. In some embodiments,

20 the composite material includes a macroporous structure (such as a macroporous mesh) having a Voronoi architecture and a biomaterial (such as a microporous biomaterial) associated with or integrated into the macroporous structure.

Embodiments of the disclosed composite materials include a macroporous structure including a plurality of open cells or pores with a Voronoi architecture. A

25 microporous biomaterial is integrated into the macroporous structure. In some embodiments, the macroporous structure is a mesh, for example, including a plurality of pores or open cells. A "macroporous structure" refers in at least some examples to a structure including pores at the μm -mm scale, such as about 10 μm to about 2 mm.

FIG. 1A shows an exemplary macroporous structure 100 that comprises a

30 plurality of fibers 102 (also referred to herein as "struts") that define a plurality of pores

104. The plurality of fibers 102 are connected at connecting points 106 that define a Voronoi architecture. The pores 104 of the macroporous structure may be any shape (such as round, rectangular, square, or polygonal). In some examples, the pores can also be of an irregular shape. In embodiments, each of the plurality of pores have the same shape (or substantially the same shape). In other embodiments, two or more of the plurality of pores in the macroporous structure have different shapes.

In some embodiments, each of the plurality of pores 104 have substantially the same shape and dimensions. In some examples, at least one dimension of the pores 104 (*e.g.*, diameter, length, or width) is at least about 0.5 mm (such as at least about 0.5 mm, at least about 0.6 mm, at least about 0.7 mm, at least about 0.8 mm, at least about 0.9 mm, at least about 1 mm, at least about 1.25 mm, at least about 1.5 mm, at least about 1.75 mm, at least about 2 mm, at least about 2.3 mm, at least about 2.5 mm, at least about 3 mm, at least about 3.5 mm, at least about 4 mm, at least about 4.5 mm, or at least about 5 mm. In other examples, at least one dimension of the pores 104 is about 0.5 mm to about 5 mm, such as about 0.5 mm to about 1.5 mm, about 1 mm to about 2 mm, about 1.5 mm to about 2.5 mm, about 2 mm to about 3 mm, about 2.5 mm to about 3.5 mm, about 3 mm to about 4 mm, about 3.5 mm to about 4.5 mm, or about 4 mm to about 5 mm. In other embodiments, at least one dimension of the pores 104 is less than about 0.5 mm (for example, less than about 0.4 mm, less than about 0.3 mm, less than about 0.2 mm, or less than about 0.1 mm). In other embodiments, the size of the pores is of a size that is sufficient for the biomaterial to enter the pores and be integrated into the macroporous structure.

In some embodiments, the size of pores is defined as the distance between the points, also referred to as “pore spacing” or the distance between each pore from the center of one pore to the center of another. In one example, pore spacing is about 3 mm. In this situation, strut thickness should be taken into account to determine pore size.

In some embodiments, the fibers 102 defining the pores 104 have a diameter of about 10 μm to about 2 mm, such as about 10 μm to about 100 μm , about 50 μm to about 500 μm , about 250 μm to about 750 μm , about 500 μm to about 1 mm, about 750

μm to about 1.25 mm, about 1 mm to about 1.5 mm, about 1.25 mm to about 1.75 mm, or about 1.5 mm to about 2 mm. In some examples, the fibers have a diameter of about 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, 1 mm, 1.1 mm, 1.2 mm, 1.3 mm, 1.4 mm, 1.5 mm, 1.6 mm, 1.7 mm, 1.8 mm, 5 1.9 mm, or 2 mm. In specific examples, the fiber diameter is about 0.7 mm, about 1 mm, or about 1.5 mm.

In other embodiments of the disclosure composite material, the macroporous structure has at least two portions wherein each of the at least two portions have different pore sizes. In some examples, this type of macroporous structure is referred to 10 herein as a “biphasic” structure. FIG. 1B shows an exemplary biphasic macroporous structure 200 including a first portion 202 and a second portion 204. In this embodiment, the pore size 206 of the first portion 202 is larger than the pore size 208 of the second portion. In some embodiments, the ratio of a dimension of the pore 206 of the first portion 202 to a dimension of the pore 208 of the second portion 208 is up to 15 about 10:1 (such as about 1.5:1, about 2:1, about 2.5:1, about 3:1, about 3.5:1, about 4:1, about 4.5:1, about 5:1, about 5.5:1, about 6:1, about 6.5:1, about 7:1, about 7.5:1, about 8:1, about 8.5:1, about 9:1, about 9.5:1, or about 10:1). In one specific example, the ratio is about 1.875:1.

The fibers 210 defining the plurality of pores in the first portion and the second 20 portion of the macroporous structure are of substantially the same size in each portion. In some embodiments, the fibers 102 defining the pores 104 have a diameter of about 10 μm to about 2 mm, such as about 10 μm to about 100 μm, about 50 μm to about 500 μm, about 250 μm to about 750 μm, about 500 μm to about 1 mm, about 750 μm to about 1.25 mm, about 1 mm to about 1.5 mm, about 1.25 mm to about 1.75 mm, or 25 about 1.5 mm to about 2 mm. In some examples, the fibers have a diameter of about 10 μm, 50 μm, 100 μm, 200 μm, 300 μm, 400 μm, 500 μm, 600 μm, 700 μm, 800 μm, 900 μm, 1 mm, 1.1 mm, 1.2 mm, 1.3 mm, 1.4 mm, 1.5 mm, 1.6 mm, 1.7 mm, 1.8 mm, 1.9 mm, or 2 mm. In specific examples, the fiber diameter is about 0.7 mm, about 1 mm, or about 1.5 mm. In some examples, the fiber diameter is substantially the same in the 30 first portion and the second portion. In other examples, the fiber diameter may be

different in the first portion and the second portion (for example, about 1:2 or about 2:1).

In other embodiments, the macroporous structure has more than two different portions (such as 3, 4, 5, or more different portions). In such structures, at least one
5 portion has a pore size that is larger than the pore size of at least one other portion of the structure. In some examples, each of the two or more portions have different pore sizes. In examples with three or more portions, each portion may have a different pore size, or at least two portions may have a substantially similar pore size. In certain
10 embodiments, any region can be defined within a shape (say rectangle) and different pore sizes can be set. For example, a rectangle could be broken down into three sections, the ends with one pore size and the middle with another. This similarly could be done with a circle, where a circle is divided into a middle region and an outer region, each with different pore sizes.

In embodiments, the macroporous structure (such as the fibers) are formed from
15 a polymer. Exemplary polymers that can be used to form the macroporous include polycaprolactone (PCL), polylactic acid (PLA), and poly lactic-co-glycolic acid (PLGA). In some examples, the polymer is biocompatible, such as a polymer that performs its desired function without eliciting undesirable local or systemic effects (such as cytotoxicity) in a subject.

20 In some examples, the amount of polymer in the composite material is an amount that does not elicit undesirable effects in a subject, such as cytotoxicity, inflammation, or an unacceptable level of inflammatory response (such as a persistent inflammatory response). In some examples, the total amount of polymer in the composite material is not more than about 30% (v/v). In other examples, the total
25 amount of the polymer in the composite material is about 5% (v/v) to about 30% (v/v), such as about 5% (v/v) to about 15% (v/v), about 10% (v/v) to about 20% (v/v), about 15% (v/v) to about 25% (v/v), or about 20% (v/v) to about 30% (v/v). In other examples, the total amount of polymer in the composite material is about 5% (v/v), about 10% (v/v), about 15% (v/v), about 20% (v/v), about 25% (v/v), about 30% (v/v),
30 about 6-8% (v/v), about 10-12% (v/v), or about 18-20% (v/v).

In some embodiments, the macroporous structure (and the resulting composite material) is a two-dimensional (or substantially two-dimensional) structure. An exemplary macroporous structure in the form of a two-dimensional sheet is illustrated in FIG. 12A. In such examples, the two-dimensional structure has a first surface (such as a top surface) and a second surface (such as a bottom surface). In other embodiments, the macroporous structure (and the resulting composite material) is a three-dimensional structure. The three-dimensional structure can be any shape, including a three-dimensional sheet (for example, as illustrated in FIG. 12A), a cube or substantially cubical (for example, as illustrated in FIG. 1A), a rectangular or substantially rectangular structure (for example, as illustrated in FIG. 1B), or a cylindrical or substantially cylindrical structure. The three-dimensional structure can also be of an irregular shape, such as a shape to conform to a bone defect in a subject.

The disclosed macroporous structures (and the resulting composite materials) can be of any size. In some examples, the macroporous structure is the size of a bone defect. In some examples, the macroporous structure is at least about 20 mm in at least one dimension, such as at least about 20-100 mm in at least one dimension, for example at least about 20 mm, at least about 25 mm, at least about 30 mm, at least about 35 mm, at least about 40 mm, 45 mm, at least about 50 mm, or more in at least one dimension. In other examples, the macroporous structure is a larger and/or non-specific size, which can be cut and shaped to the desired size and shape for treating a subject with a bone defect.

In some embodiments, the macroporous structure is isotropic, for example, having the same properties in all axes. In other embodiments, the macroporous structure is anisotropic, for example, having different properties in at least one axis compared to at least one other axis. Material anisotropy may arise intentionally (for example, by stretching the Voronoi mesh in one axes to induce structural alignment) or may be due to the material design (*e.g.*, planar materials where one direction (such as thickness) is comprised of only a few pores).

The stiffness of the disclosed composite materials can be tuned based on the density of the pores in the macroporous structure. For example, a macroporous

structure with a higher density of pores (for example, smaller pores defined by more closely placed connecting points of the fibers) will have higher stiffness or lower deformability. A macroporous structure with a lower density of pores (for example, larger pores defined by more distantly placed connecting points of the fibers) will have lower stiffness or higher deformability. In some embodiments, the Young's modulus of the macroporous structure is between about 100 kPa and about 250 MPa, for example from about 100 kPa to about 500 kPa, about 250 kPa to about 750 kPa, about 500 kPa to about 1 MPa, about 1 MPa to about 5 MPa, about 2.5 MPa to about 10 MPa, about 7.5 MPa to about 15 MPa, about 10 MPa to about 20 MPa, about 15 MPa to about 40 MPa, about 25 MPa to about 50 MPa, about 30 MPa to about 75 MPa, about 50 MPa to about 100 MPa, about 100 MPa to about 150 MPa, about 125 MPa to about 175 MPa, about 150 MPa to about 200 MPa, about 175 MPa to about 225 MPa, or about 200 MPa to about 250 MPa. In one embodiment of a biphasic material, a "porous" region could have a Young's modulus of about 1-5 MPa and a "dense" region could have a Young's modulus of about 10-40 MPa.

In additional embodiments, the macroporous structure is capable of elastic deformation for up to 20% of applied strain, such as up to 5%, up to 10%, up to 15%, or up to 20% of applied strain. Elastic deformation refers to reversible or temporary deformation. In other embodiments, the macroporous structure is capable of plastic deformation for up to 80% of applied strain, such as up to 5%, up to 10%, up to 15%, up to 20%, up to 25%, up to 30%, up to 35%, up to 40%, up to 45%, up to 50%, up to 55%, up to 60%, up to 65%, up to 70%, up to 75%, or up to 80% of applied strain. Plastic deformation refers to permanent or non-reversible deformation.

The disclosed composite materials include a microporous biomaterial that is integrated into or associated with the macroporous structure. A "microporous biomaterial" refers to a structure including pores at the nm- μ m scale, such as about 100 nm to about 10 μ m. In some examples, the biomaterial is not covalently linked to the macroporous structure. In other embodiments, the biomaterial is covalently linked to the macroporous structure, for example by a linker. In one example, covalent linkage includes crosslinking of collagen fibers to the support structure, such as using EDC-

NHS chemistry or other chemicals which can covalently link the end groups of collagen to the end groups of the 3D-printed materials. In some embodiments, the biomaterial substantially fills all of the porous space in the macroporous structure (see, *e.g.*, FIG. 6C, part 2). In other embodiments, the biomaterial does not completely fill all of the porous space in the macroporous structure (see, *e.g.*, FIG. 6C, part 1).

In some embodiments, the biomaterial includes one or more components that are biocompatible and are suitable for use in repair of a bone defect. In some examples, the biomaterial includes collagen, glycosaminoglycans, calcium phosphate, or a combination of two or more thereof. In one example, the biomaterial is mineralized collagen, for example, produced by combining collagen with calcium phosphate during fabrication of the composite (see Section II, below). In some examples, the biomaterial includes other extracellular matrix materials (which contain collagen), and may also include minerals, glycosaminoglycans, and other chemicals or additives (honey, growth factors, proteins, etc.).

II. Methods of Making the Composite Materials

Methods of making the disclosed composite materials are provided herein. In some embodiments, the methods include contacting a macroporous structure (such as that described in Section I) with a solution including a liquid suspension of a biomaterial. The liquid is then removed from the solution in contact with the macroporous structure, producing the composite material.

In some embodiments, contacting the macroporous structure with the solution includes submerging the macroporous structure in the solution. The liquid can be removed by cooling the macroporous structure and solution to a temperature at which the liquid transforms into a plurality of solid crystals and removing at least some (or substantially all) of the crystals by sublimation or evaporation. In some examples, the liquid is removed by lyophilization. In one example, the suspension is lyophilized by reducing the temperature to about -10°C at a rate of about 1°C/minute and holding at -10°C for a sufficient period of time for sublimation of the crystals, for example about 2 hours. The resulting composite material is brought back to room temperature and

atmospheric pressure. In some examples, the pressure is released and temperature is increased rapidly, for example over about 2-3 minutes.

In other embodiments, other lyophilization conditions include altering the hold temperature (-40°C, -60°C, etc.), altering the rate of freezing (for example, slower (such as 1°C/2 min) or faster (such as 5°C/min), which can result in different pore architectures of the resulting material.

In some embodiments, the solution includes a liquid suspension of collagen (for example, about 2% w/v collagen). In some examples, the collagen is type I collagen. The collagen may be human type I collagen, bovine type I collagen, or xenografts (such as porcine dermis, for example for wound healing). The solution may also contain calcium and phosphorus, for example, for producing mineralized collagen. In some examples, the solution includes about 2% w/v type I collagen, about 40% w/v mineral solution of phosphoric acid and calcium hydroxide. Additional components that can be added to the solution include zinc (see, *e.g.*, Tiffany *et al.*, *Acta Biomaterialia* 93:86-92, 2019), growth factors (such as BMP2, see *e.g.*, Tiffany *et al.*, *RSC Advances* 10:26982, 2020), glycosaminoglycans, such as chondroitin (for example, 0.5-1% v/v chondroitin sulfate sodium salt), heparin or heparan sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid, or amniotic membrane from placenta (see, *e.g.*, Kolliopoulos *et al.*, *Frontiers in Bioengineering and Biotechnology* 10:1034701, 2022). Calcium nitrate tetrahydrate can also be added.

The methods also include preparing the macroporous structure in some embodiments. The macroporous structure can be produced by any suitable method, including additive manufacturing (*e.g.*, 3D printing) methods or laser sintering. In some examples, the methods also include designing a Voronoi structure with the desired characteristics, for example by selecting the distance between the connecting points of the fibers defining the pores or the macroporous structure (“point spacing”) and the diameter of the fibers. The design can be carried out using commercially available software packages, such as nTop software (nTopology, New York) or Fusion360 software (Autodesk, California). In some examples, a generative design process is used to produce a Voronoi architecture of random shape. For example, a program may allow

for the user to set a point-spacing of Voronoi design across a user-set print size, such as a rectangle, and the program will design the Voronoi outline to fill this space. The user can then select the strut thickness of the print. The macroporous structure can be produced from a design by additive manufacturing, such as extrusion printing (*e.g.*,
5 fused deposition modeling). In some examples, standard 3D-printing, photolithograph/stereolithography, and/or laser sintering can be used to produce the macroporous structure.

In some embodiments, the composite material can be sterilized. Once produced, the composite material can be stored until ready to use. In some examples, the
10 composite material is stored in a desiccator at room temperature. When ready to use, the composite material can be rehydrated, for example, by soaking in a sterile solution, such as saline, Ringer's solution, or another physiologically suitable fluid. In some examples, the solution may include one or more biomolecules, such as one or more growth factors (*e.g.*, BMP2 or VEGF), honey, or antibiotics.

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III. Methods of Treating Bone Defects

The composite materials described herein may be used to treat a bone defect in a subject. In embodiments, the methods include implanting a disclosed composite material in, or at the site of, a bone defect in a subject. The composite material is
20 surgically implanted at the site of a bone defect in order to treat the bone defect. In some examples, the material is fastened in place (such as with a fastener or adhesive). In other examples, the material is conformally fitted and does not require fastening. The subject may be a human or animal subject (such as a laboratory animal, companion animal, or livestock).

25 In some embodiments, the composite material is sized to fit the defect to be repaired. In other embodiments, the methods include shaping the composite material to the bone defect prior to implanting in the subject. In some examples, a composite material (such as a previously produced composite material) is trimmed to the size and shape of the bone defect in the subject. In other examples, a composite material is
30 designed specifically to fit the bone defect of a subject and is produced using the

methods described herein. In the case of a composite material specifically designed to fit a subject's bone defect, some trimming and/or shaping may be needed prior to implanting to improve the fit of the composite in the defect. In additional embodiments, a two-dimensional or three-dimensional composite material may be used for treating a bone defect, depending on the location or type of defect. For example, a two-dimensional composite material may be used to treat a defect of the eye orbital or a cranial defect, while a three-dimensional composite material may be used to treat a defect of the jaw.

In some embodiments, the composite material deforms to fit the defect. In particular examples, the composite material includes a biphasic macroporous structure such that it includes a region of capable of high deformation, which improves conformal fitting (for example at one or more edges of the structure) and a region of little or no deformation, which provides increased mechanical support and stability due to increased stiffness (for example in a central region of the structure).

In additional embodiments, the methods of treating a bone defect include hydrating a previously produced composite material in a sterile solution prior to implanting the composite in the bone defect in the subject. In other embodiments, the methods further include designing and producing a composite material having characteristics (such as Voronoi architecture, size, and shape) selected for the particular bone defect to be treated.

In some embodiments, a bone defect includes a disease, defect, or disorder which affects bone strength, function, and/or integrity, such as those resulting from injury or trauma, or a defect brought about during the course of surgery, infection, malignancy, or developmental malformation. Examples of bone defects include, but are not limited to, fractures (such as a critical defect or non-union fracture) and craniomaxillofacial defects (such as cleft palate or facial or dental injuries or malformations), non-union fractures, or for treatment of any portion of missing bone (such as bone removed by resection, such as for osteosarcoma). In some embodiments, the bone defect is a defect, fracture, or injury of one or more cranial bones, such as the frontal bone, parietal bone, temporal bone, occipital bone, sphenoid bone, or ethmoid

bone or one or more facial bones, such as the zygomatic bone, superior and inferior maxilla, nasal bone, mandible, palantine bone, lacrimal bone, vomer bone, or the inferior nasal conchae.

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EXAMPLES

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

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Example 1

Materials and Methods

Three objectives were pursued in these studies (FIG. 2B): (1) Alter the thickness and porosity of Voronoi designs to determine how changes in this relate to a predictive equation and stiffness changes and examine isotropy to determine if the direction of force applied on these designs results in different stiffnesses; (2) Create biphasic Voronoi structures to determine if stiffness and strain can be localized to one region and how these integrate with mineralized collagen scaffolds; and (3) Test the shape-fitting ability of 2D and 3D Voronoi “sheets” and how these integrate with mineralized collagen scaffolds and how these can be cut and trimmed to shape.

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Fabrication of 3D-printed Voronoi structures: All 3D Voronoi structures were designed using nTop software (nTopology, New York, USA) and 2D designs were designed using Fusion360 (Autodesk, California, USA). Point spacing was defined as the distance (mm) between the randomly seeded points within a Voronoi structure.

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Fabrication of photopolymerized Voronoi structures: Voronoi structures made of photopolymerized resin were created for two different objectives, 1 and 2. Objective 1 structures measured 10 mm cubes with three different porosities and were created by altering the Voronoi point spacing (4 mm point spacing, 3.5 mm point spacing, 3 mm point spacing) with 0.7 mm strut diameter. The porosity was further minimized by printing 0.5 mm strut diameter prints with 4 mm point spacing. These .stl files were added to a Form 2 photopolymerization printer (Formlabs, Massachusetts,

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USA). A white standard resin and a Dental SG resin (Formlabs) were used to create all samples for objective 1. Dental resin was cured post-print with a Form Cure (Formlabs). Objective 2 structures were made of white resin, and these designs were fabricated as 20 x 6 x 6 mm (length, width, height) Voronoi rectangular prints with a point spacing of 2 on one half of the structure and a point spacing of 3.75 on the other half, and printed with a strut thickness of 0.7 mm diameter. These were compared to uniform Voronoi rectangles, measuring the same dimensions and a point space of 2 throughout the space.

Fabrication of laser-sintered Voronoi structures: Voronoi structures used for objective 1 and 3 were created by laser sintering poly(caprolactone). The Voronoi structures were manufactured using laser sintering with an EOS Formiga P110. These structures were fabricated using a blend of polycaprolactone (PCL), a bioresorbable and biodegradable polyester, with a 4% weight by weight ratio of hydroxyapatite, a naturally occurring mineral. Voronoi structures for objective 1 were fabricated as 25 mm cubes with the same pore spacing (3 mm) and various strut thicknesses (0.7 mm, 1 mm, 1.5 mm). Voronoi structures for objective 3 were fabricated as either 2D or 3D sheets, with 2D sheets measuring 74 x 74 mm squares with 245 cells and 80% cell scale, and 3D sheets measuring 74 x 74 x 6 mm (length, width, height) and 3 point spacing and 0.7mm strut thickness.

Fabrication of Voronoi-mineralized collagen composite structures: Voronoi-mineralized collagen composites were fabricated for objectives 2 and 3. Mineralized collagen suspension was created and composites were fabricated via lyophilization. Briefly, 1.9 w/v% type I bovine collagen (Collagen Matrix, New Jersey, USA or Sigma Aldrich, Missouri, USA) was blended together with a 40 wt% mineral solution of phosphoric acid (Fisher Scientific, New Hampshire, USA) and calcium hydroxide (Sigma Aldrich), 0.84 v% chondroitin sulfate sodium salt (CAS 9082-07-9, Spectrum Chemicals, New Jersey, USA), and calcium nitrate tetrahydrate (Sigma Aldrich) using a rotor-stator in a jacketed cooling vessel until well-blended. To create mineralized collagen scaffolds, 24 mL or 48 mL of suspension (for 3.5 mm or 7.5 mm thickness sheets, respectively) was pipetted into a 75 x 75 mm aluminum pan. To

create biphasic or uniform Voronoi-mineralized collagen composites for objective 2, 48 mL of suspension was added to a 75 x 75 mm aluminum pan and individual rectangular 3D-prints were carefully added to pan, making sure to cover the print with suspension. To create Voronoi-mineralized collagen composite sheets for objective 3, 24 mL or 48 mL of suspension (for 2D or 3D composites, respectively) was pipetted into a 75 x 75 mm aluminum pan and Voronoi 3D-printed structures were carefully added to this with tweezers, making sure to cover the 3D-prints with the suspension. The suspensions were then lyophilized using a Genesis freeze-dryer (VirTis, New York, USA) by dropping the temperature at a rate of 1°C/min to -10°C, then holding these at -10°C for 2 hours, and then these were brought back to room temperature and atmospheric pressure. Resulting mineralized collagen and composites were in the form of solid sheets. For objective 2, composites were removed by a razor blade. For objective 3, composites were removed with 12 mm biopsy punches.

Predictive equation for Voronoi structures: A predictive modulus equation for open-cell foams was used to predict the Elastic Modulus of Voronoi structures.

$$\frac{E^*}{E_s} = \left(\frac{\rho^*}{\rho_s} \right)^2$$

Where E^* represents the Predictive Young's Modulus, E_s represents the Young's Modulus of the solid skeleton, and (ρ^*/ρ_s) represents the reduced density. The predictive modulus values were determined by calculating the v/v% of Voronoi designs based on .stl files (known densities) and normalizing these to the design with the greatest volume of 3D-print (i.e. thickest or most dense print). Thus the Voronoi architecture with the highest relative density was the "base" (value of 1) design, and was used to calculate the expected modulus of the remaining Voronoi architectures with smaller relative densities based on the known differences in relative density.

Compression testing of Voronoi structures: All Voronoi structures created underwent compression testing and were analyzed with a custom Matlab program in order to determine Young's Modulus, Ultimate Stress, and Ultimate Strain as open porous foam structures. All structures were loaded onto platens facing the same

direction (same face upward). To test isotropy, each design was tested on each face of the cube (x, y, z axis). Matching experimental data to a predictive equation was determined by normalizing the other designs tested to the design with the highest density of 3D-print.

5 **Compression of Voronoi structures for objective 1:** Photopolymerized resin Voronoi structures printed as 10 mm cubes were mechanically compressed by an Instron 5943 mechanical tester (Instron, Massachusetts, USA). A 100 N load cell was used at a rate of 1 mm/min to generate data for stress-strain curves. Six samples were used for each design and each face tested for 10 mm cube designs. Laser-sintered
10 polycaprolactone Voronoi structures printed as 25 mm cubes were mechanically loaded and unloaded to half the thickness of the print. A MTS Criterion model 43 (MTS, Minnesota, USA) was used to compress at a rate of 1 mm/sec with a 1 kN load cell and holding at 1 sec before releasing at a rate of 1 mm/sec. Videos of prints were taken using a Pixelink camera (Pixelink, Canada). Only the 0.7 mm thickness print was used
15 to test for isotropy. Percent deformation of samples was determined by the difference in the strain after unloading compared to the strain at the start of the experiment. Eight samples were used for each thickness and face tested for 25 mm cube designs.

Compression and DIC testing of Voronoi structures for objective 2:

Biphasic Voronoi structures were compressed by an Instron 5943 mechanical tester
20 (Instron) with a 100 N load cell at a rate of 3 mm/min to generate data for stress-strain curves. 3D-printed Voronoi-mineralized collagen composites were tested with a biphasic 3D-print and a uniform 3D-print (n=6). Rectangular scaffolds were glued to 3D-printed bases (ABS) to stabilize these to the mechanical platens, with the dense region of the biphasic structure always glued to the base. Following embedding into the
25 ABS base, scaffolds were speckle-patterned with waterproof India ink (BLICK Art Materials, Illinois, USA) using a gravity feed airbrush with a nozzle size of 0.3 mm (Got Hobby Inc., California, USA). During uniaxial compression testing, images were taken with a Canon EOS 5DS R DLSR camera and a Canon Macro 100-mm lens at a rate of 1 image every 5 seconds until an arbitrary point of maximum compression
30 (Canon, Tokyo, Japan). The digital images were correlated. Briefly, the sets of images

taken during compression testing were correlated using a version of the MATLAB file package “Digital Image Correlation and Tracking” (Copyright © 2010, C. Eberl, D.S. Gianola, S. Bundschuh) modified by Elizabeth Jones (Improved Digital Image Correlation version 4 – Copyright © 2013, 2014, 2015 by Elizabeth Jones) to calculate
5 local strain across scaffolds. For each set of scaffold images, the region of interest was set as the entire exposed scaffold and full images were correlated (subset size: 221-421; threshold 0.5; search zone: 2; grid step size: 25). Finally, displacements were smoothed prior to calculating strains to reduce noise (Gaussian distribution of weights; kernel size: 11; number of smoothing passes: 3; maximum size of contiguous non-correlated
10 points to smooth over: 15) and local strains were calculated using a cubic (16-node) algorithm. Due to high displacements, images near the maximum compression did not correlate. Therefore, strain profiles were analyzed from the start of compression to simply show the localization of strain throughout the scaffold in biphasic versus uniform Voronoi structures. Scaffolds without any 3D-print, biphasic Voronoi-
15 mineralized collagen composites, and uniform Voronoi-mineralized collagen composites were tested to analyze elastic modulus regimes from stress-strain curves using the same mechanical tester, a 100 N load cell at a compression rate of 3 mm/min.

Push-out testing of Voronoi structures and composites for objective 3: 2D and 3D Voronoi-mineralized collagen sheets underwent push-out testing to determine
20 shape-fitting ability compared to mineralized collagen scaffolds without printed supports. 12 mm biopsy punches were used to remove samples from sheets, and these were added to two defect sizes in a Teflon mold, either 11.5 mm or 10.8 mm in diameter. The Teflon mold was applied to an apparatus allowing for a metal pin to push the composites or scaffolds through the defects. The maximum push out force (N) was
25 measured and compared between groups, with eight samples per group. 2D Voronoi composites were compared to mineralized collagen scaffolds of the same thickness (approximately 3.5 mm thick) and 3D Voronoi composites were compared to mineralized collagen scaffolds of the same thickness (approximately 7.5 mm thick).

SEM imaging of Voronoi and mineralized collagen composites: The 3D-
30 print integration with mineralized collagen scaffolds was visualized via SEM of

photopolymerized biphasic Voronoi-mineralized collagen composites, and 2D and 3D Voronoi-mineralized collagen composites. Biphasic composites were cut in both phases to expose the interior of the dense and porous regions, and 2D and 3D Voronoi composites were cut in half to visualize the center of these composites.

- 5 **Statistics:** Data were first checked for normality (Shapiro-Wilk) and equal variance (Levene's Test) of residuals, using a Grubb's test to check for and remove any outliers. If data were normal with equal variance, a One-way ANOVA was used with a Tukey post-hoc to determine significance ($p < 0.05$) between more than 2 groups. For only two group comparisons, a two sample T-test was used, and data was marked only
10 significant if the power was above 0.8.

Example 2

Analysis of Young's Modulus and Isotropy

- Four different porosity Voronoi structures were tested for stiffness (19.43 v/v%,
15 14.74 v/v%, 11.82 v/v%, 6.2 v/v%). The Young's Moduli of these four different porosities of Voronoi designs was evaluated with prints of either white or dental photopolymerized resin. Overall, the Young's Moduli of 0.7 mm strut diameter Dental resin 3D prints were greater than those of the white print (Table 1). Denser Voronoi structures also led to higher Young's Moduli, and if printed with white
20 photopolymerized resin, these matched a predictive modulus closely (FIG. 3B, Table 2).

Table 1. Comparison of the Young's modulus of different porosity Voronoi 3D-prints made of white or dental resin

		Dental Resin	White Resin
Sample	Side	Young's Modulus (MPa)	Young's Modulus (MPa)
0.5mm dia 4 pore	1	0.19 ± 0.13	0.13 ± 0.05
	2	0.38 ± 0.09	0.18 ± 0.04
	3	0.14 ± 0.13	0.09 ± 0.07

		Dental Resin	White Resin
Sample	Side	Young's Modulus (MPa)	Young's Modulus (MPa)
0.7mm dia 4 pore	1	4.12 ± 0.91	1.98 ± 0.16
	2	12.4 ± 1.92	1.35 ± 0.17
	3	8.94 ± 0.96	1.00 ± 0.18
0.7mm dia 3.5 pore	1	5.91 ± 2.76	2.35 ± 0.26
	2	7.00 ± 2.80	2.39 ± 0.16
	3	10.5 ± 2.27	1.65 ± 0.23
0.7mm dia 3 pore	1	11.6 ± 3.10	4.35 ± 0.07
	2	11.4 ± 1.77	3.49 ± 0.44
	3	9.02 ± 0.77	2.51 ± 0.33

Table 2. Determination of predictive modulus and experimental modulus of Voronoi 3D prints made of either white or Dental resin based on point spacing and strut thickness.

Predictive Equation					Experimental data (dental resin)		Experimental Data (white resin)	
Design	v/v%	ρ^*/ρ_s	$(\rho^*/\rho_s)^2$	Normalized E*	Young's Modulus (MPa)	Normalized E*	Young's Modulus (MPa)	Normalized E*
4	6.22	0.06	0.004	0.11	0.14	0.01	0.17	0.05
3	11.82	0.12	0.014	0.37	8.48	0.80	1.42	0.40
2	14.74	0.15	0.022	0.58	7.82	0.73	1.93	0.54
1	19.43	0.19	0.038	1.00	10.66	1.00	3.57	1.00

5

The Young's Moduli of three different thicknesses (0.7 mm, 1 mm, 1.5 mm) of Voronoi designs printed with laser-sintered polycaprolactone were compared. The Young's Modulus of each thickness was significantly ($p < 0.05$) different from one another, with the 1.5 mm, 1 mm, and 0.7 mm thicknesses having an average of 20.64

MPa, 5.86 MPa, and 1.02 MPa, respectively (Table 3, FIG. 4). When compared to the predictive modulus of these designs, the experimental values closely matched (FIG. 4, Table 4). Compression and release of samples to determine deformation demonstrated that with an increase in thickness, there was an increase in deformation without recovery (Table 5, FIG. 5B). Additionally, videos of compressive loading and unloading of 3D-prints demonstrated the flexibility of prints, with the 0.7 mm strut thickness print showing buckling behavior (not shown).

Table 3. Comparison of Young's modulus, ultimate stress and strain, and % deformation after loading of 25 mm dia. Voronoi cubes printed of PCL in three different diameter struts (0.7, 1, and 1.5 mm, Objective 1). Additionally, the 0.7 mm cube design was compressed on each of its 3 faces (x, y, z) to determine changes in these properties.

Sample	Young's Modulus (MPa)	Ultimate Stress (MPa)	Ultimate Strain (mm/mm)	Deformation after Loading (%)
Side 1	0.74 ± 0.05	0.04 ± 0.003	0.07 ± 0.006	2.95 ± 0.69
Side 2	1.02 ± 0.05	0.05 ± 0.003	0.09 ± 0.004	3.97 ± 0.23
Side 3	1.01 ± 0.06	0.05 ± 0.004	0.09 ± 0.002	3.61 ± 0.41
0.7 mm	1.02 ± 0.05	0.05 ± 0.003	0.09 ± 0.004	3.97 ± 0.23
1.0 mm	5.86 ± 0.36	0.31 ± 0.01	0.10 ± 0.002	5.58 ± 0.68
1.5 mm	20.64 ± 1.23	1.15 ± 0.07	0.12 ± 0.002	10.57 ± 0.18

Table 4. Determination of predictive modulus and experimental modulus of Voronoi 3D prints made of polycaprolactone based on strut thickness.

Predictive Equation					Experimental Data	
Design (mm)	$v/v\%$	ρ^*/ρ_s	$(\rho^*/\rho_s)^2$	Normalized E^*	Young's Modulus (MPa)	Normalized E^*
0.7	7.72	0.08	0.01	0.06	1.02	0.05
1	14.47	0.14	0.02	0.22	5.86	0.28

1.5	30.96	0.31	0.10	1.00	20.64	1.00
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Table 5. Comparison of Young's modulus, ultimate stress and strain, and % deformation after loading of 25 mm dia. Voronoi cubes printed of PCL in three different diameter struts (0.7, 1, and 1.5 mm, Objective 1). Additionally, the 0.7 mm cube design was compressed on each of its 3 faces (x, y, z) to determine changes in these properties.

Sample	Young's Modulus (MPa)	Ultimate Stress (MPa)	Ultimate Strain (mm/mm)	Deformation after Loading (%)
Side 1	0.74 ± 0.05	0.04 ± 0.003	0.07 ± 0.006	2.95 ± 0.69
Side 2	1.02 ± 0.05	0.05 ± 0.003	0.09 ± 0.004	3.97 ± 0.23
Side 3	1.01 ± 0.06	0.05 ± 0.004	0.09 ± 0.002	3.61 ± 0.41
0.7 mm	1.02 ± 0.05	0.05 ± 0.003	0.09 ± 0.004	3.97 ± 0.23
1.0 mm	5.86 ± 0.36	0.31 ± 0.01	0.10 ± 0.002	5.58 ± 0.68
1.5 mm	20.64 ± 1.23	1.15 ± 0.07	0.12 ± 0.002	10.57 ± 0.18

Isotropy of various Voronoi designs was investigated by compressing the three faces (x, y, z axis) of resin and polycaprolactone 3D-prints. Both Dental and white resin prints demonstrated anisotropy regardless of print porosity or thickness (Table 6). Compression of the 0.7 mm thickness polycaprolactone 3D print also demonstrated anisotropy, as the Young's Modulus and amount of deformation was dependent on what side was compressed (FIG. 7A and 7B).

Table 6. Comparison of significance between Young's Modulus of various sides tested of white and Dental resins. x represents the sides are not significantly ($p < 0.05$) different from one another. * represents the sides are significantly ($p < 0.05$) different from one another.

Design	Side	Dental Resin			White Resin		
		1	2	3	1	2	3

0.5 mm dia. 4 pore	1		*	X		X	X
	2	*		*	X		X
	3	X	*		X	X	
0.7mm dia. 4 pore	1		X	X		*	*
	2	X		*	*		*
	3	X	*		*	*	
0.7mm dia. 3.5 pore	1		X	*		X	*
	2	X		X	X		*
	3	*	X		*	*	
0.7mm dia. 3 pore	1		X	X		*	*
	2	X		*	*		X
	3	X	*		*	X	

Example 3

Biphasic Voronoi Structures

Mineralized collagen scaffolds were combined with biphasic Voronoi 3D-prints as well as uniform Voronoi 3D-prints made of white photopolymerized resin. During compression of biphasic 3D-prints without collagen, there was a porous 3D-print compressive region and a dense 3D-print compression region visible on stress-strain curves (FIG. 8A). Within each of these dense and porous print regions, an elastic regime, collapsed plateau, and densification regime was visible (FIG. 8B). SEM analysis demonstrated that in the more porous regions of the biphasic composite material, there was good integration between the 3D-print and collagen scaffold (FIG. 9). In the denser regions of the biphasic composite material, there were some empty spaces where collagen was unable to fully penetrate due to the small pores and high density of the dense region of the 3D-print. A similar dense and porous 3D-print moduli regime was visible in the biphasic Voronoi-mineralized collagen composites, and the addition of mineralized collagen added an increased Young's Modulus in the porous and dense region compared to the 3D-print alone (FIGS. 8A and 8B). Additionally, it

was more difficult to predict the moduli of the rectangular Voronoi 3D-print, as the experimentally determined porous region moduli of the biphasic print was much higher than the predictive moduli (FIGS. 9A-9D). DIC testing of the uniform and biphasic Voronoi composites demonstrated that biphasic composites had strain localization in the porous region only, while uniform composites had strain localization randomly throughout the structure (FIG. 10). This region of strain was also more intense (higher strain), in the biphasic composite than the uniform composite.

Example 4

Analysis of Push-out Force

2D and 3D Voronoi composite sheets were compared to mineralized collagen scaffolds in a push-out test with two defect sizes (11.5 mm and 10.8 mm). Both the 2D and 3D Voronoi 3D-prints were well integrated into the surrounding mineralized collagen scaffold with no empty spaces or holes (FIG. 12A, FIG. 13). 12 mm cylindrical specimens were added to two smaller cylindrical defects and the maximum force achieved to push these through a Teflon mold was measured. There were no differences in push-out force between the mineralized collagen scaffold and the 2D Voronoi composite (FIG. 12B). Additionally, both the mineralized collagen scaffold and 2D Voronoi composite did not require significantly ($p < 0.05$) greater force to push through the 11.5 mm defect and the 10.8 mm defect. The 3D Voronoi composite did have significantly ($p < 0.003$, $p < 0.00005$) greater force required to push it through the 11.5 mm defect and 10.8 mm defect, respectively, than the mineralized collagen scaffold. Additionally, pushing the 3D Voronoi composite through the 10.8 mm defect required significantly ($p < 0.02$) greater force than the 11.5 mm defect. Although mineralized collagen scaffolds were tested with two different thicknesses to better compare to the dimensions of the 2D and 3D Voronoi composites, there were no significant ($p < 0.05$) differences in the push-out force required of these different thicknesses in the 11.5 or 10.8 mm defects. To note, the 2D Voronoi composites were more difficult to add to the Teflon mold due their stiff nature compared to the 3D Voronoi composites, which were easily deformable.

Example 5

In vivo Testing of Composite Material

This example describes experiments for testing use of a disclosed composite material to repair a bone defect in an *in vivo* rabbit model. One skilled in the art will appreciate that methods that deviate from these specific methods can also be used to treat a bone defect *in vivo*.

Approximately three weeks prior to creation of the parietal defect, 26 female New Zealand White rabbits (2–3 months old) undergo bone marrow harvest from the ilium. Preoperatively, rabbits are injected subcutaneously with enrofloxacin (5 mg/kg) and acepromazine (1 mg/kg). Anesthesia is maintained with isoflurane gas (1.5–3%) during the procedure and pain control is performed with buprenorphine 0.05mg/kg and carprofen 4mg/kg subcutaneous injection. Isolated BMSCs are cultured and 2×10^6 cells are seeded onto scaffolds in proliferation media. 24 hours after seeding, proliferation media is exchanged for osteogenic differentiation media consisting of 10 mM β -glycerophosphate, 50 μ g/ml ascorbic acid and 0.1 μ M dexamethasone. Scaffolds are untreated or treated with rhBMP-2 at a concentration of 50 ng/mL for one week. Media and BMP-2 are changed every 2–3 days.

The head of each rabbit is shaved and disinfected with Betadine. The cranial surface is exposed by a midline incision and the overlying parietal periosteum is dissected off of the calvarium. For each rabbit, a 14 mm full thickness, extradural defect is created by a hand powered trephine and the bone is lifted away without injury to the dura. One scaffold is implanted for each rabbit and the incision is closed with sutures.

Twelve weeks after implantation, the rabbits are euthanized by intravenous injection of 1 mL of pentobarbital solution (Fatal-Plus®, 390 mg/ml) intravenously via the marginal ear vein. The previous incision is then reopened and the calvarium is exposed. The calvarium including the cranial defect is analyzed grossly and then explanted for micro-CT, histologic, and biomechanical analyses. Gene expression and “push-out” testing may also be performed.

In view of the many possible embodiments to which the principles of the disclosure may be applied, it should be recognized that the illustrated embodiments are only examples and should not be taken as limiting the scope of the invention. Rather,
5 the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A composite material comprising:
a macroporous structure having a Voronoi architecture; and
5 a microporous biomaterial integrated into the macroporous structure.
2. The composite material of claim 1, wherein the macroporous structure comprises a mesh comprising a plurality of pores.
- 10 3. The composite material of claim 1 or claim 2, wherein the mesh comprises pores of at least about 0.5 mm in at least one dimension.
4. The composite material of claim 4, wherein the pores are about 0.5 mm to about 5 mm in at least one dimension.
- 15 5. The composite material of any one of claims 1 to 4, wherein the macroporous structure comprises fibers connecting points defining the Voronoi architecture.
6. The composite material of claim 5, wherein the fibers have a diameter of about
20 10 μ m to about 2 mm.
7. The composite material of any one of claims 1 to 6, wherein the macroporous structure comprises at least a first portion and a second portion, and wherein the pore size of the first portion is greater than the pore size of the second portion.
- 25 8. The composite material of claim 7, wherein the ratio of the pore size of the first portion to the second portion is up to about 10:1.

9. The composite material of any one of claims 1 to 8, wherein the macroporous structure is a two-dimensional structure having a first surface and a second surface.

10. The composite material of any one of claims 1 to 8, wherein the macroporous
5 structure is a three-dimensional structure.

11. The composite material of any one of claims 1 to 10, wherein the macroporous structure is isotropic or anisotropic.

10 12. The composite material of any one of claims 1 to 11, wherein the macroporous structure has a Young's modulus between 100 kPa and 250 MPa.

13. The composite material of any one of claims 1 to 12, wherein the macroporous structure is capable of elastic deformation for up to 20% of applied strain, plastic
15 deformation for up to 80% strain, or both.

14. The composite material of any one of claims 1 to 13, wherein the biomaterial comprises collagen, glycosaminoglycans, calcium phosphate, or a combination of two or more thereof.

20

15. The composite material of claim 14, wherein the collagen is mineralized collagen.

16. The composite material of any one of claims 1 to 15, wherein the biomaterial is
25 not covalently linked to the macroporous structure.

17. The composite material of any one of claims 1 to 15, wherein the biomaterial is covalently linked to the macroporous structure.

30

18. A method of making a composite material of any one of claims 1 to 17,
comprising:

contacting the macroporous structure with a solution comprising a liquid
suspension of a biomaterial; and

5 removing the liquid from the solution.

19. The method of claim 18, wherein contacting the macroporous structure with the
solution comprises submerging the macroporous structure in the solution.

10 20. The method of claim 17 or claim 18, wherein removing the liquid from the
solution comprises:

cooling the solution in contact with the macroporous structure to a temperature
at which the liquid transforms into a plurality of solid crystals; and

15 removing at least some of the plurality of solid crystals by sublimation and/or
evaporation.

21. The method of any one of claims 17 to 20, wherein the biomaterial comprises
collagen, glycosaminoglycans, calcium phosphate, or a combination of two or more
thereof.

20

22. The method of any one of claims 17 to 21, further comprising preparing the
macroporous structure.

23. The method of claim 22, wherein preparing the macroporous structure
25 comprises one or more of designing the macroporous structure and producing the
macroporous structure by additive manufacturing or laser sintering.

24. The method of claim 23, wherein designing the macroporous structure
comprises utilizing a generative design process to produce a Voronoi architecture of
30 random shape.

25. A method of treating a bone defect, comprising implanting the composite material of any one of claims 1 to 17 in a bone defect in a subject.

5 26. The method claim 25, wherein the bone defect is a craniomaxillofacial bone defect.

27. The method of claim 25 or claim 26, further comprising shaping the composite material to the bone defect prior to implanting the composite material in the subject.

10

28. The method of any one of claims 25 to 27, further comprising hydrating the composite material in a sterile solution prior to implanting in the subject.

COMPOSITE MATERIALS AND METHODS OF MAKING AND USING THE SAME

5

ABSTRACT OF THE DISCLOSURE

Composite materials including a macroporous structure having a Voronoi
architecture and a microporous biomaterial integrated into the macroporous structure
are provided. Methods of making the composite materials and methods of using the
10 materials to treat a bone defect are also provided.

FIG. 1A

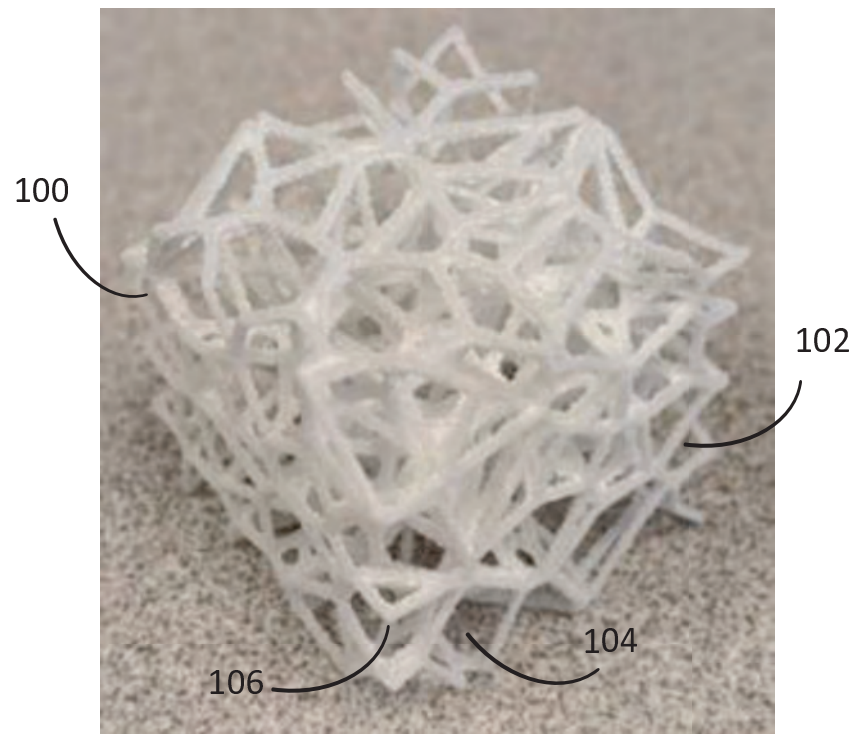


FIG. 1B

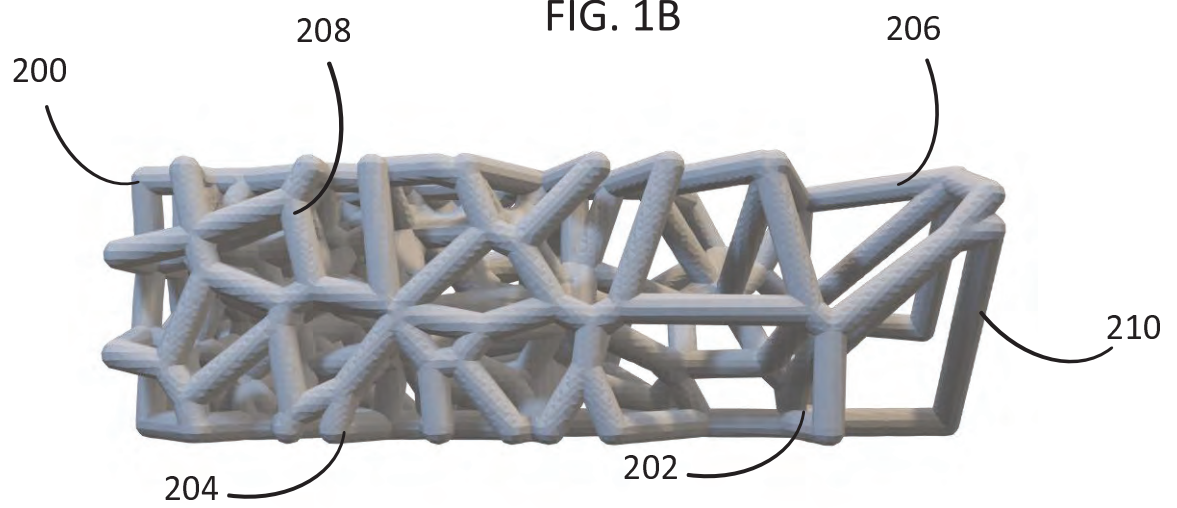


FIG. 2A

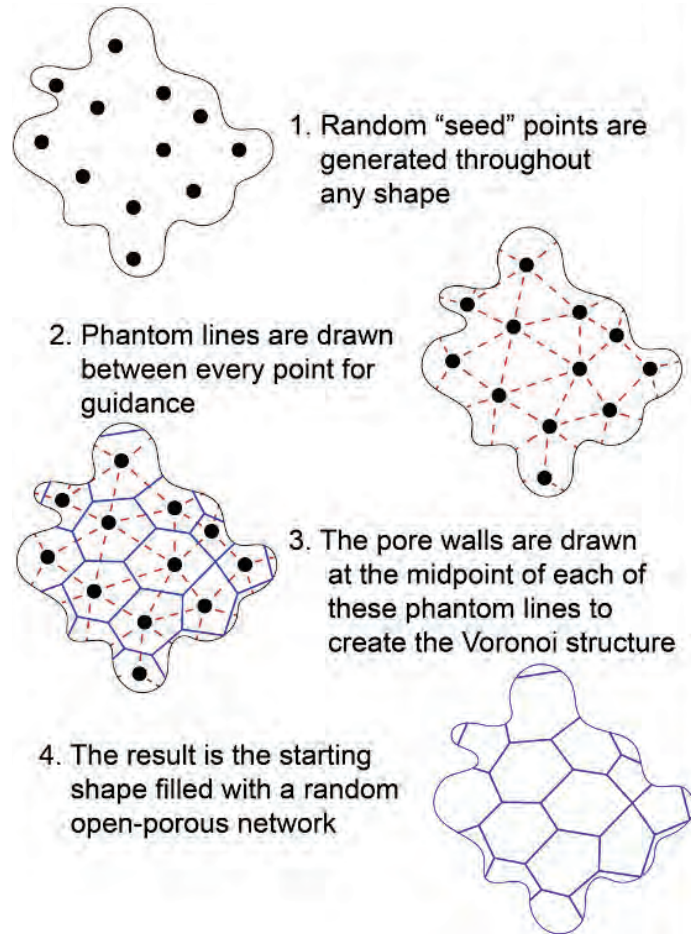
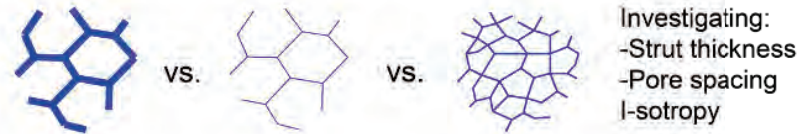
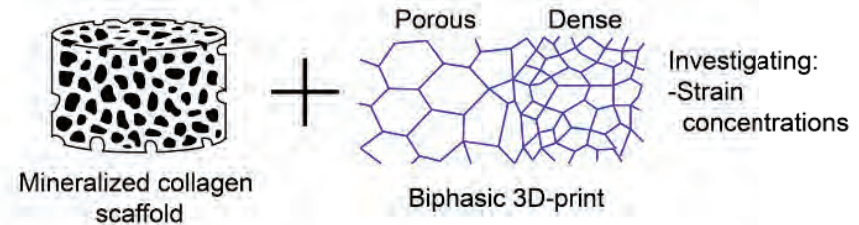


FIG. 2B

Objective 1: Alter the porosity of Voronoi structures to achieve various mechanical properties



Objective 2: Create mineralized collagen scaffolds reinforced with biphasic Voronoi 3D-prints



Objective 3: Evaluate shape-fitting and osteogenesis of 2D and 3D Voronoi composite sheets

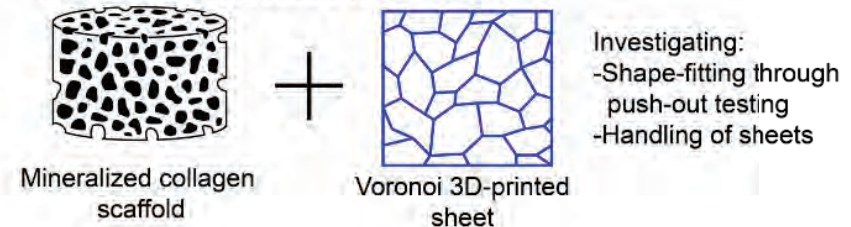


FIG. 3A

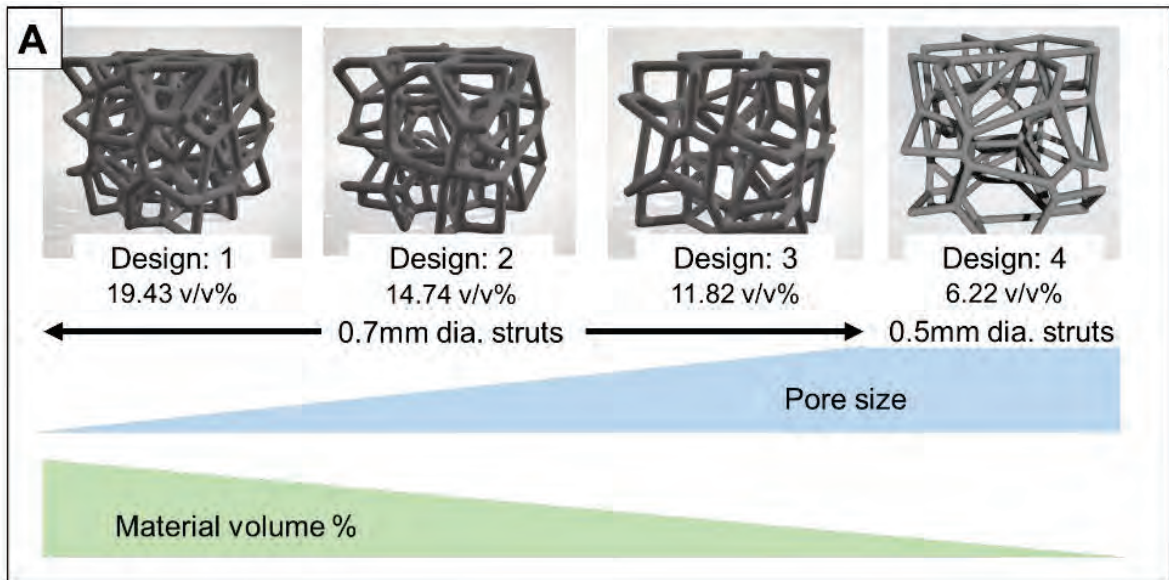


FIG. 3B

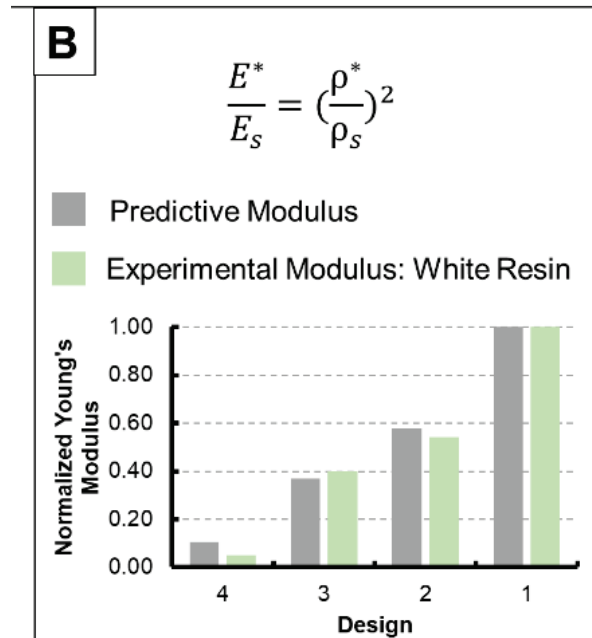


FIG. 4

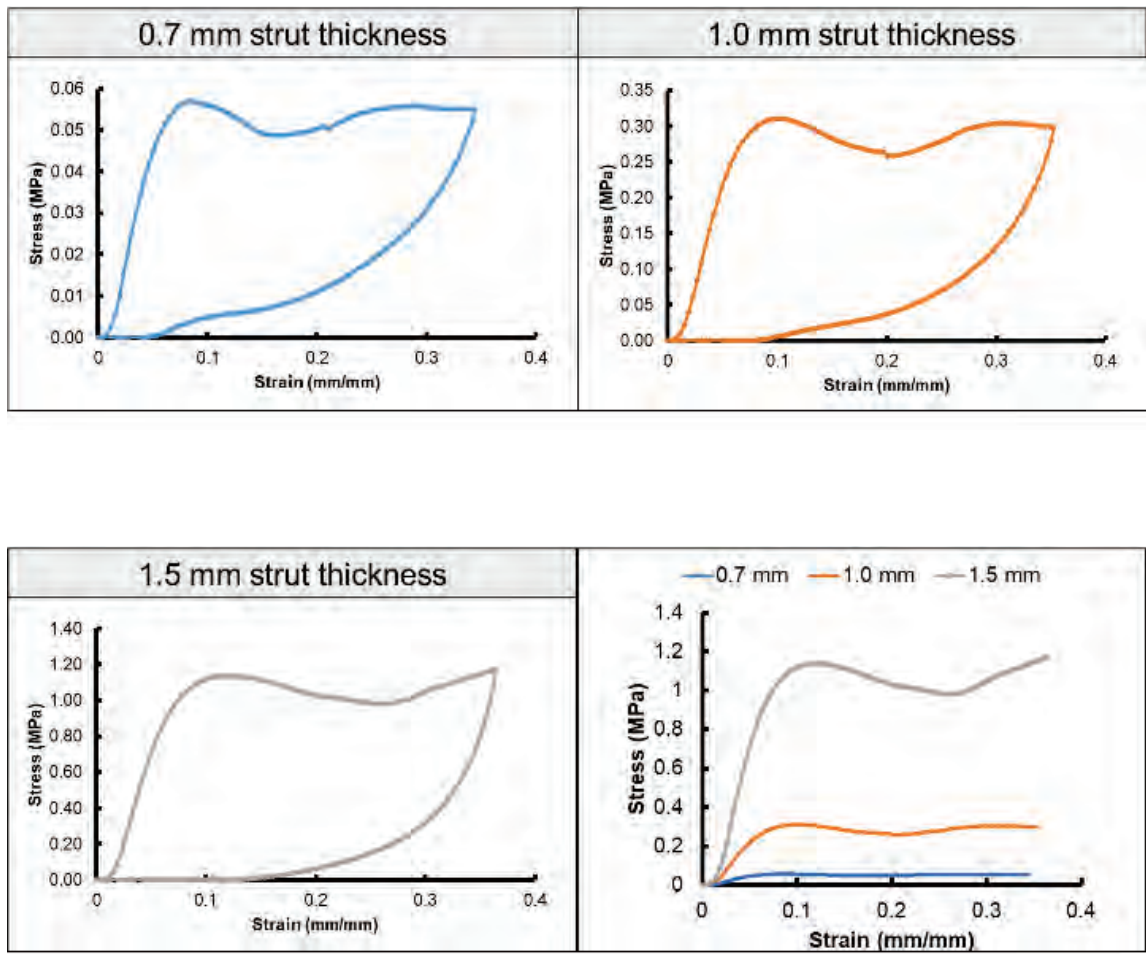


FIG. 5A

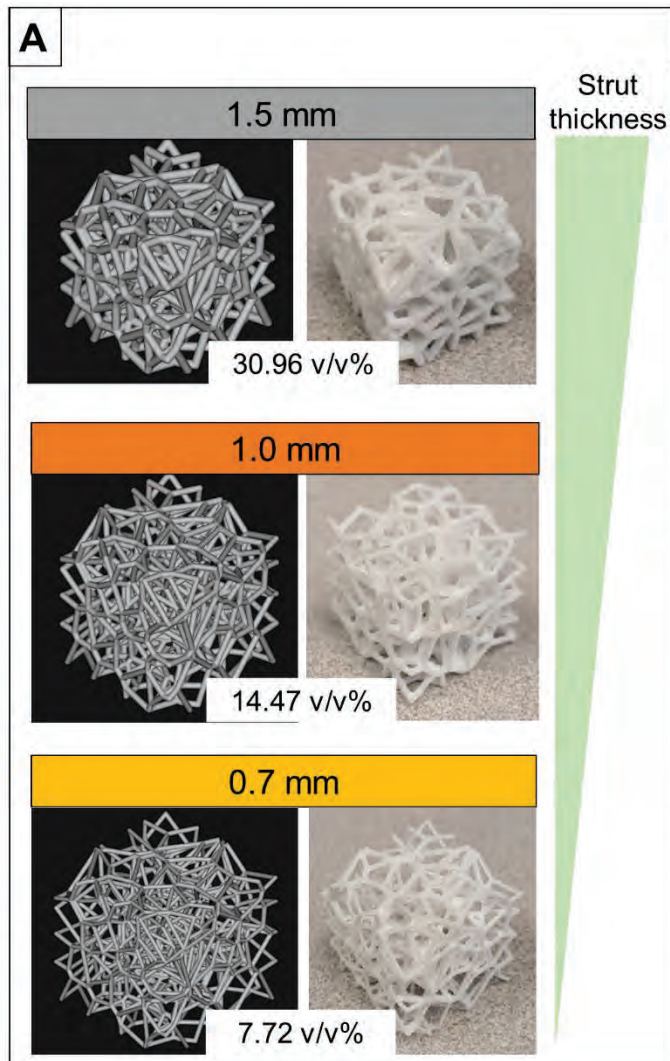


FIG. 5B

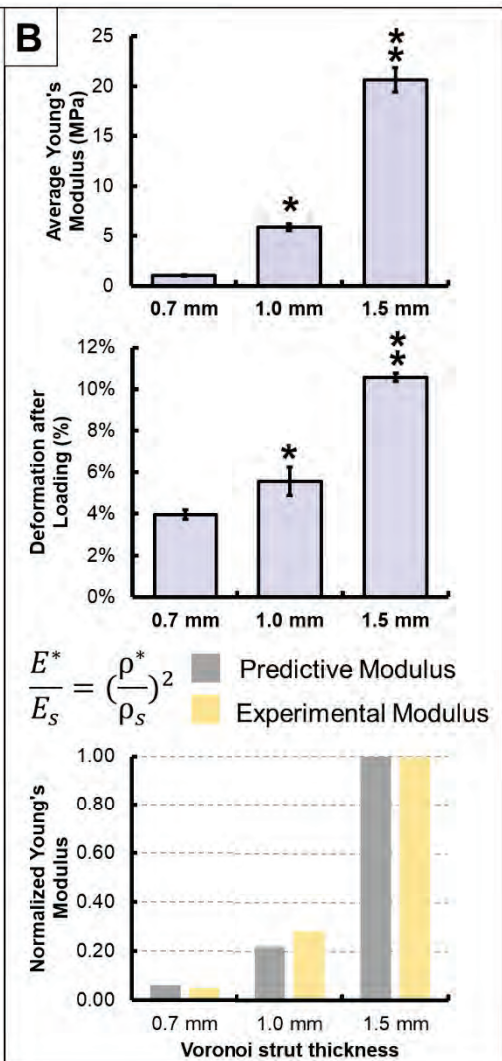


FIG. 6A

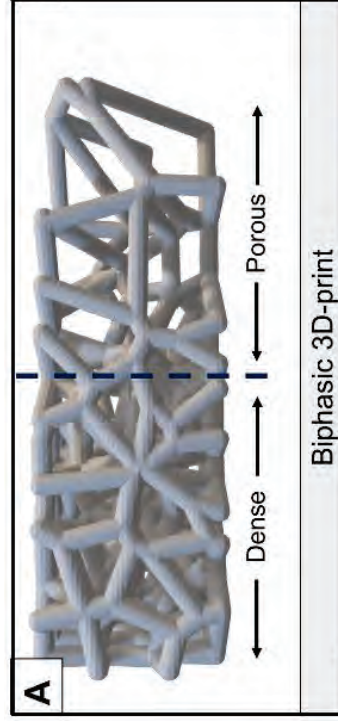


FIG. 6B



C



FIG. 6C

FIG. 7A

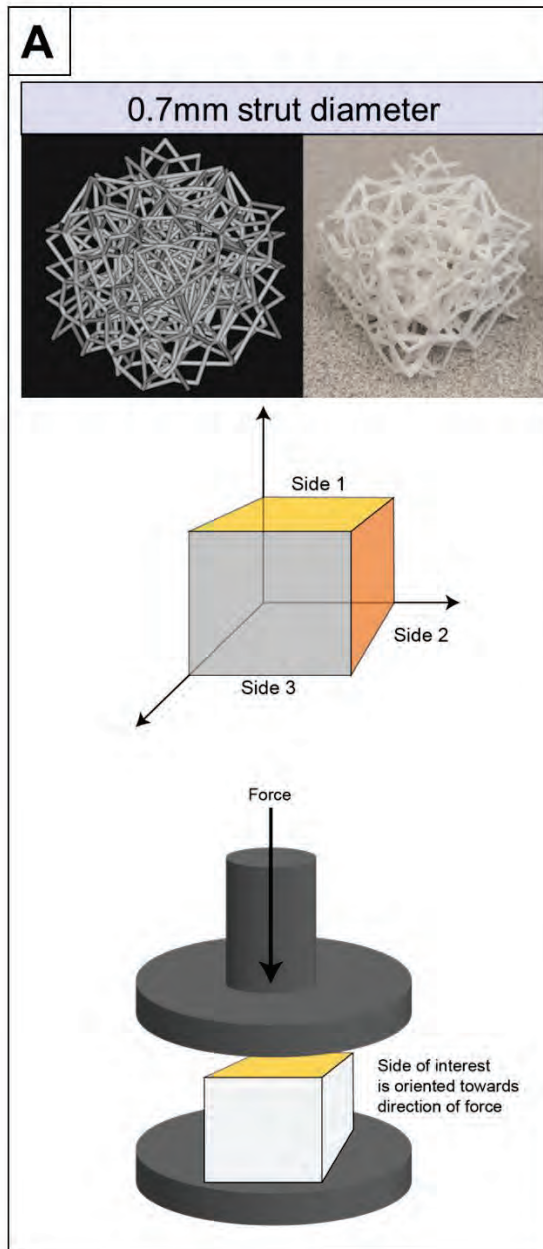


FIG. 7B

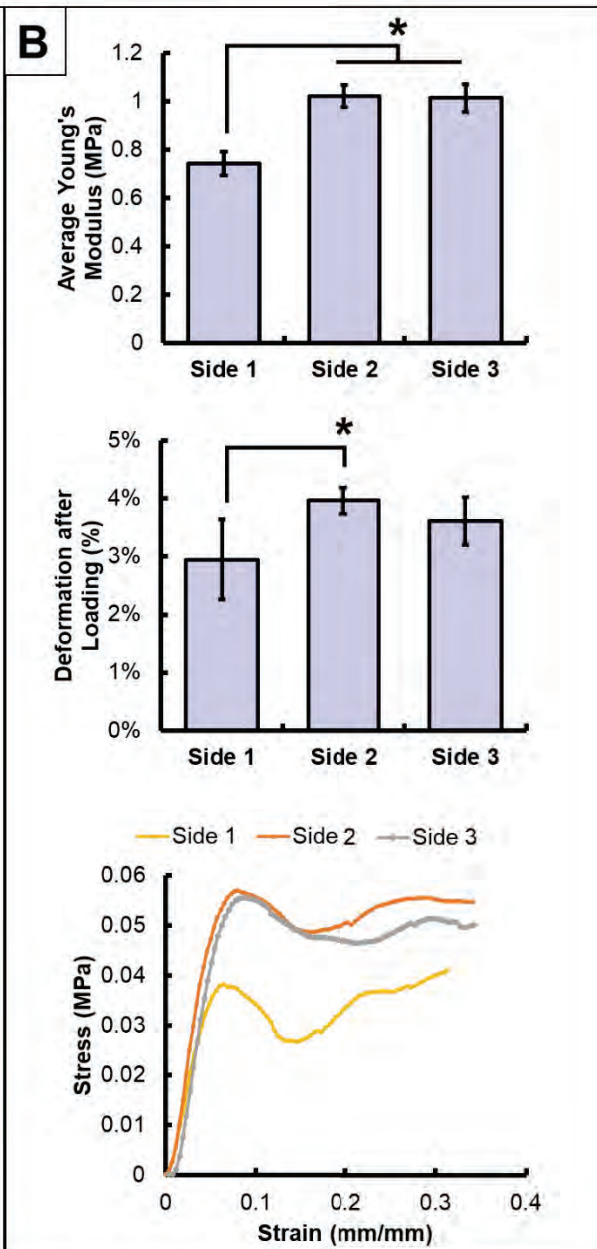


FIG. 8A

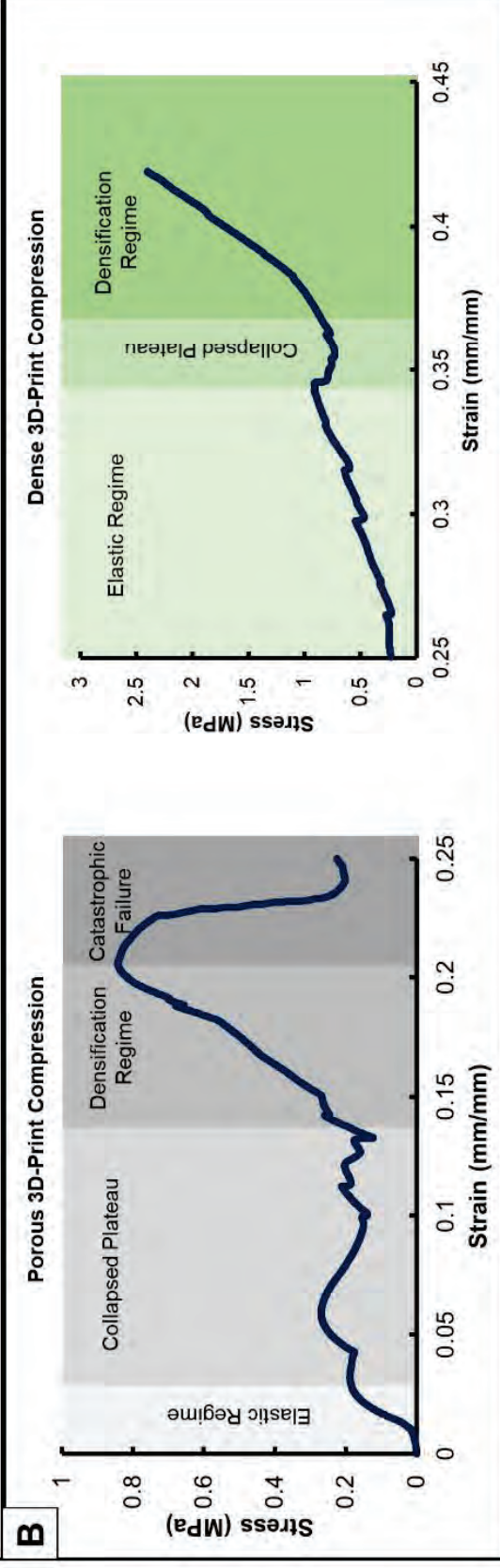
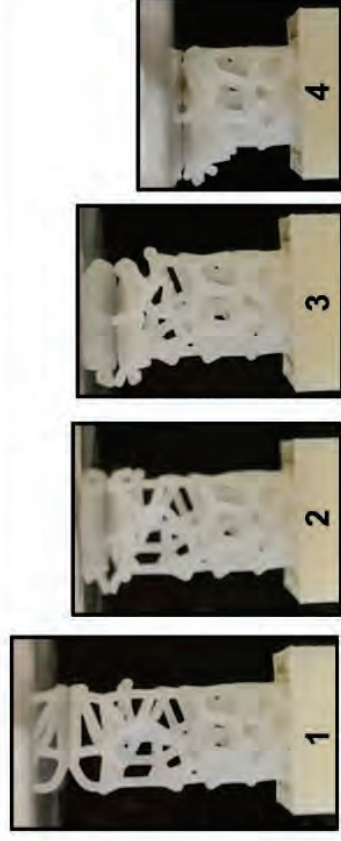
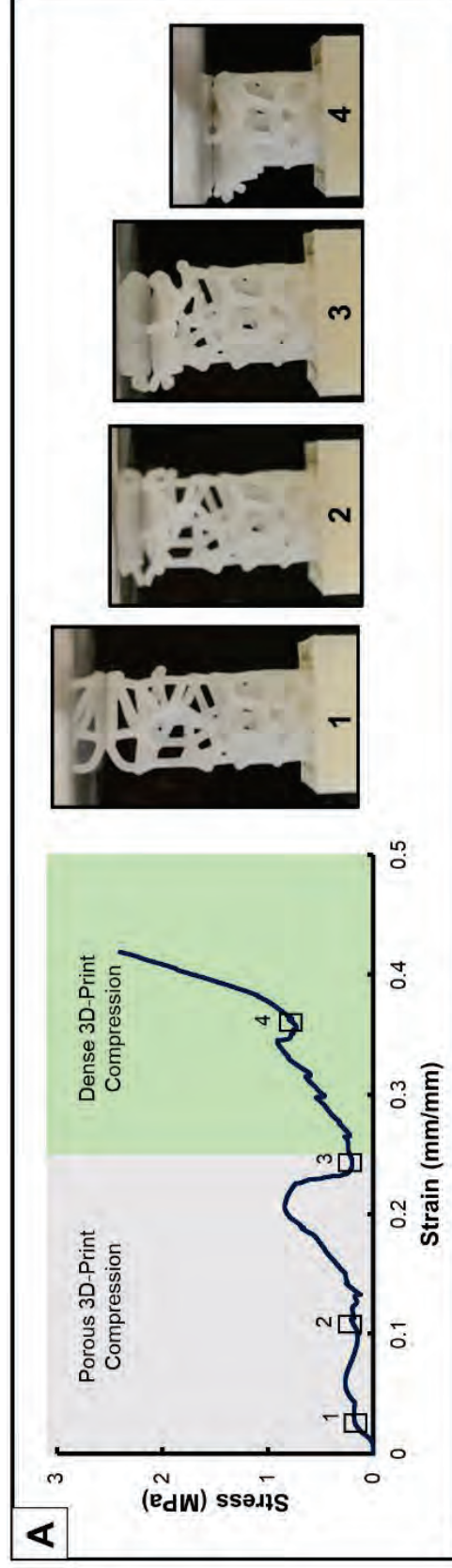


FIG. 8B

FIG. 9A

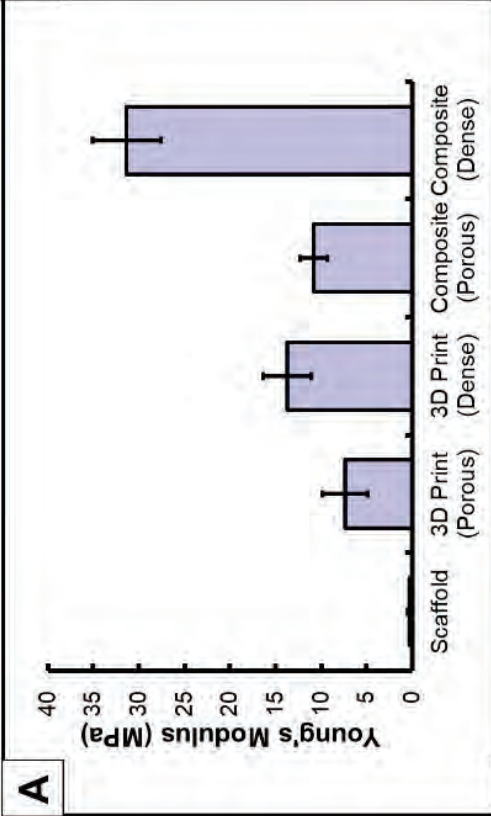


FIG. 9B

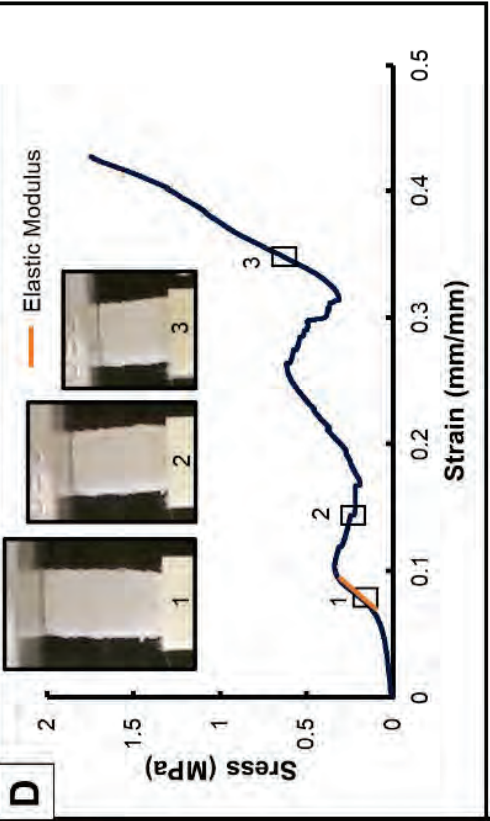
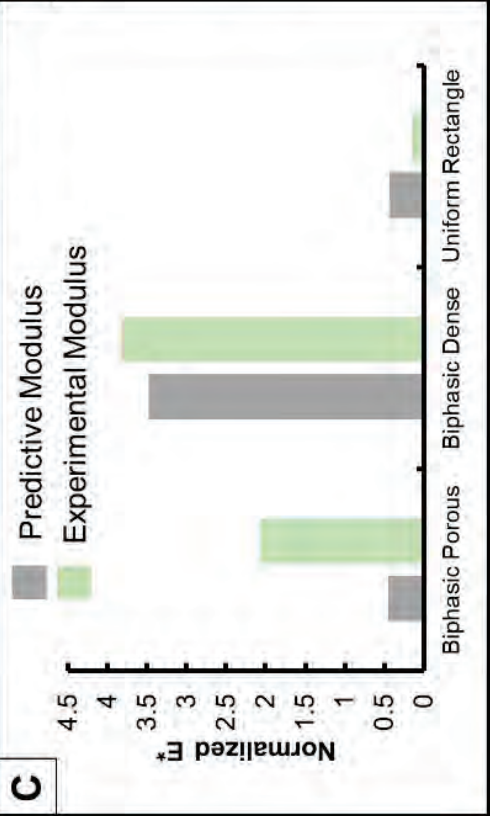
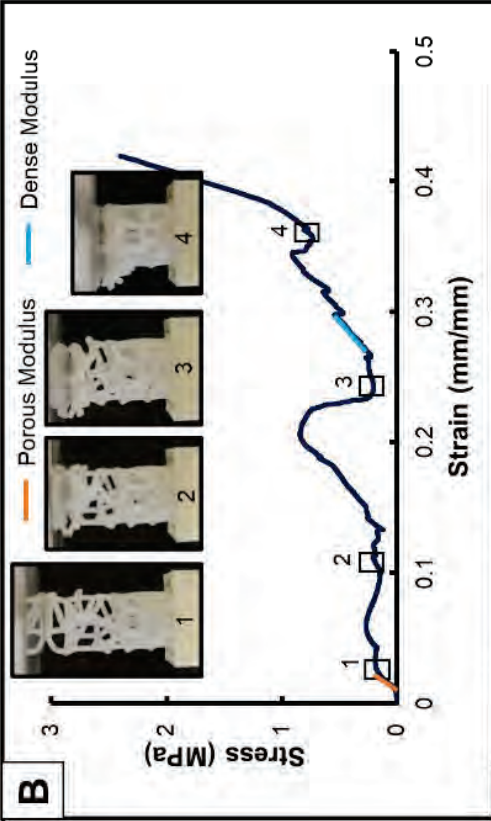


FIG. 9C

FIG. 9D

FIG. 10

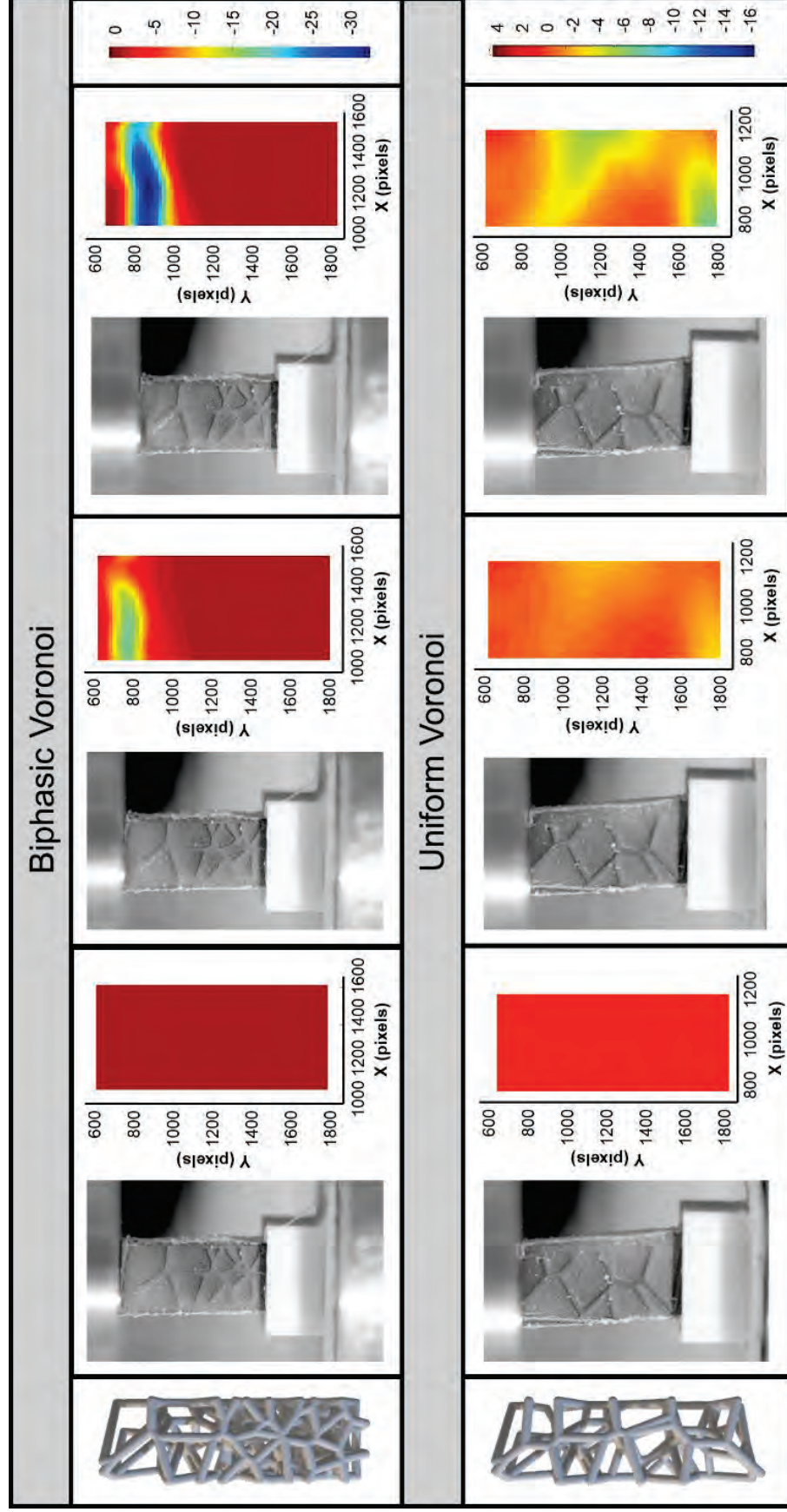


FIG. 11

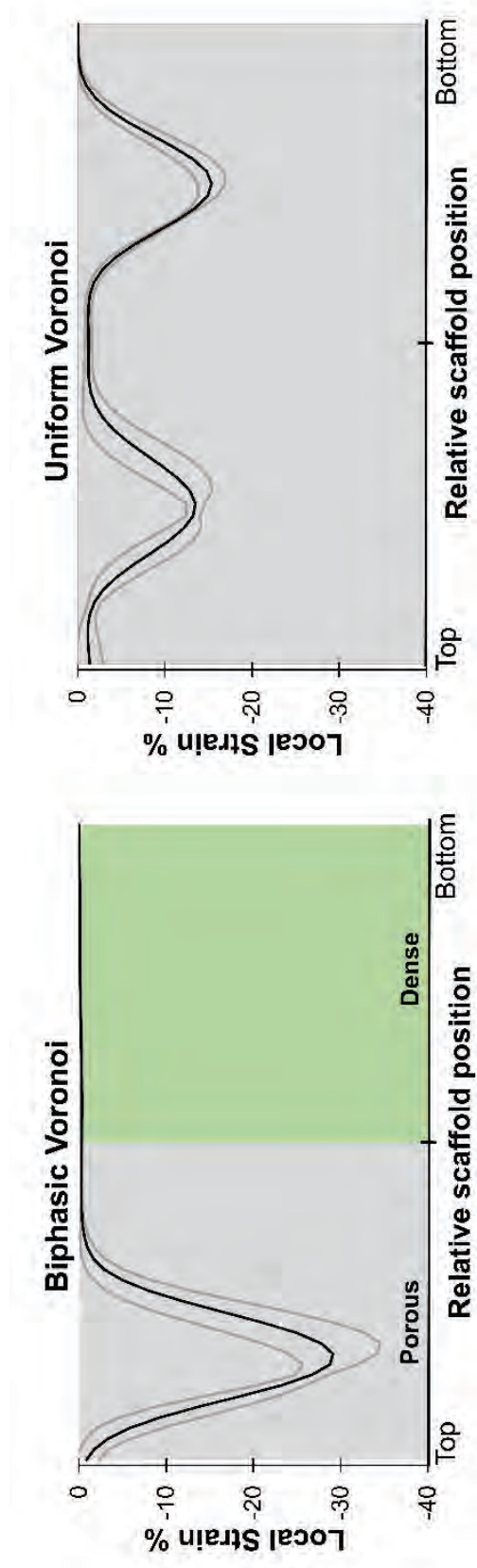


FIG. 12A

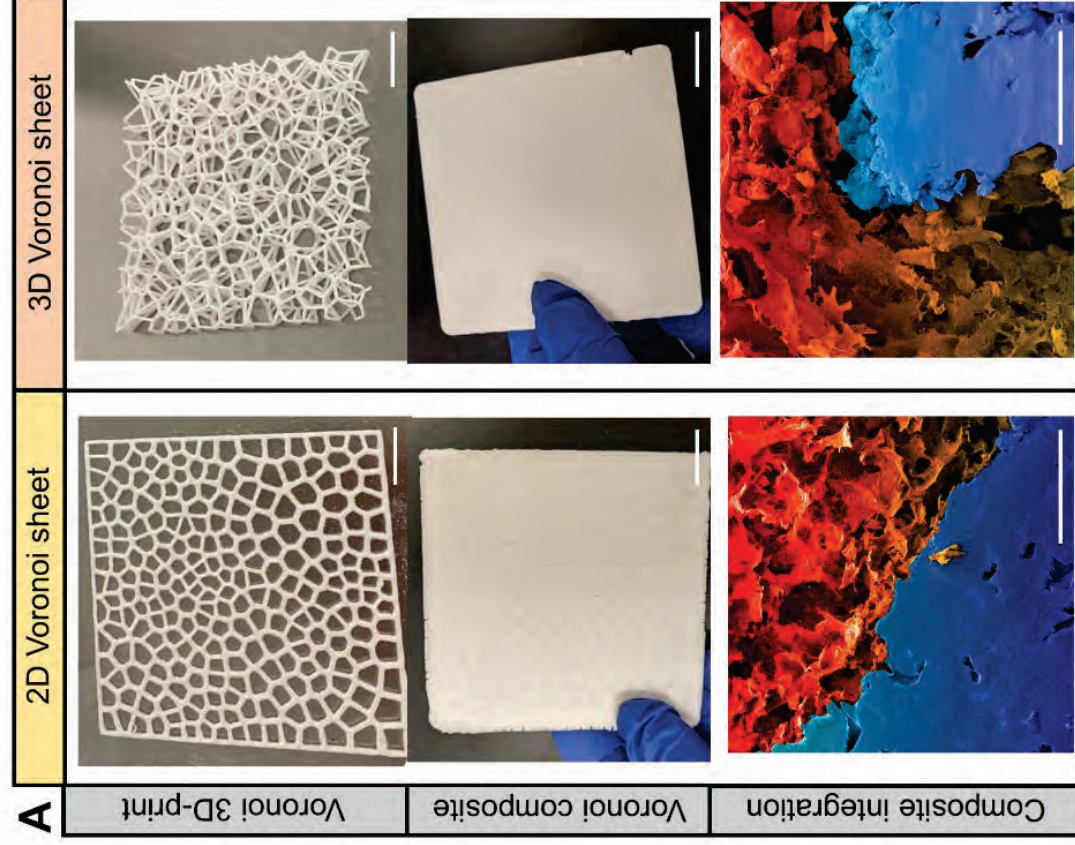


FIG. 12B

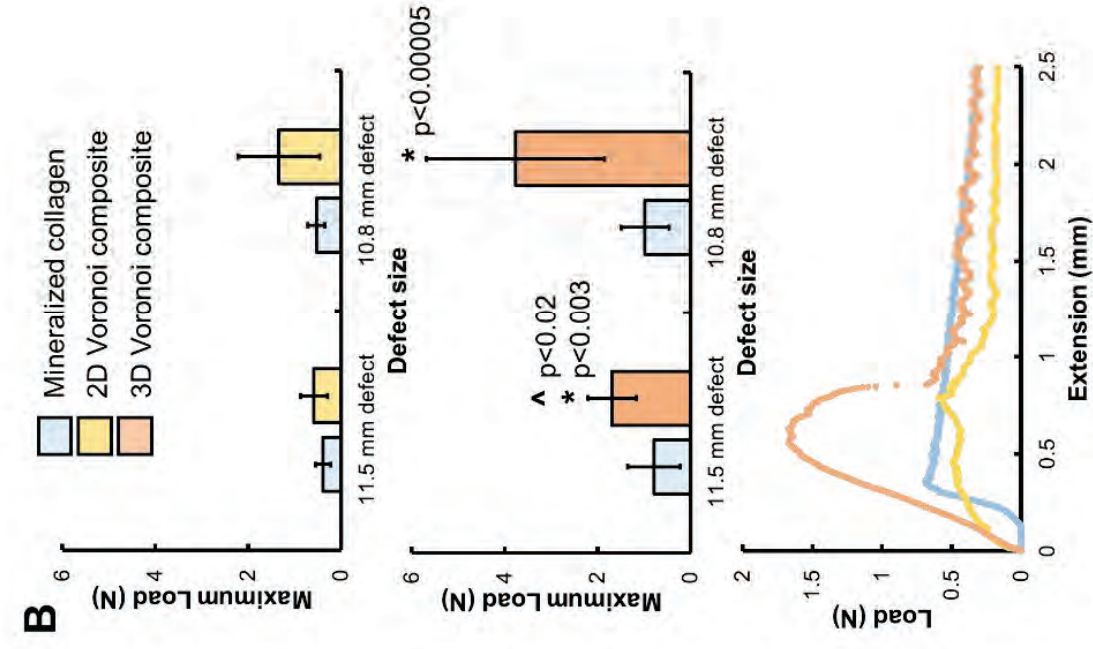


FIG. 13

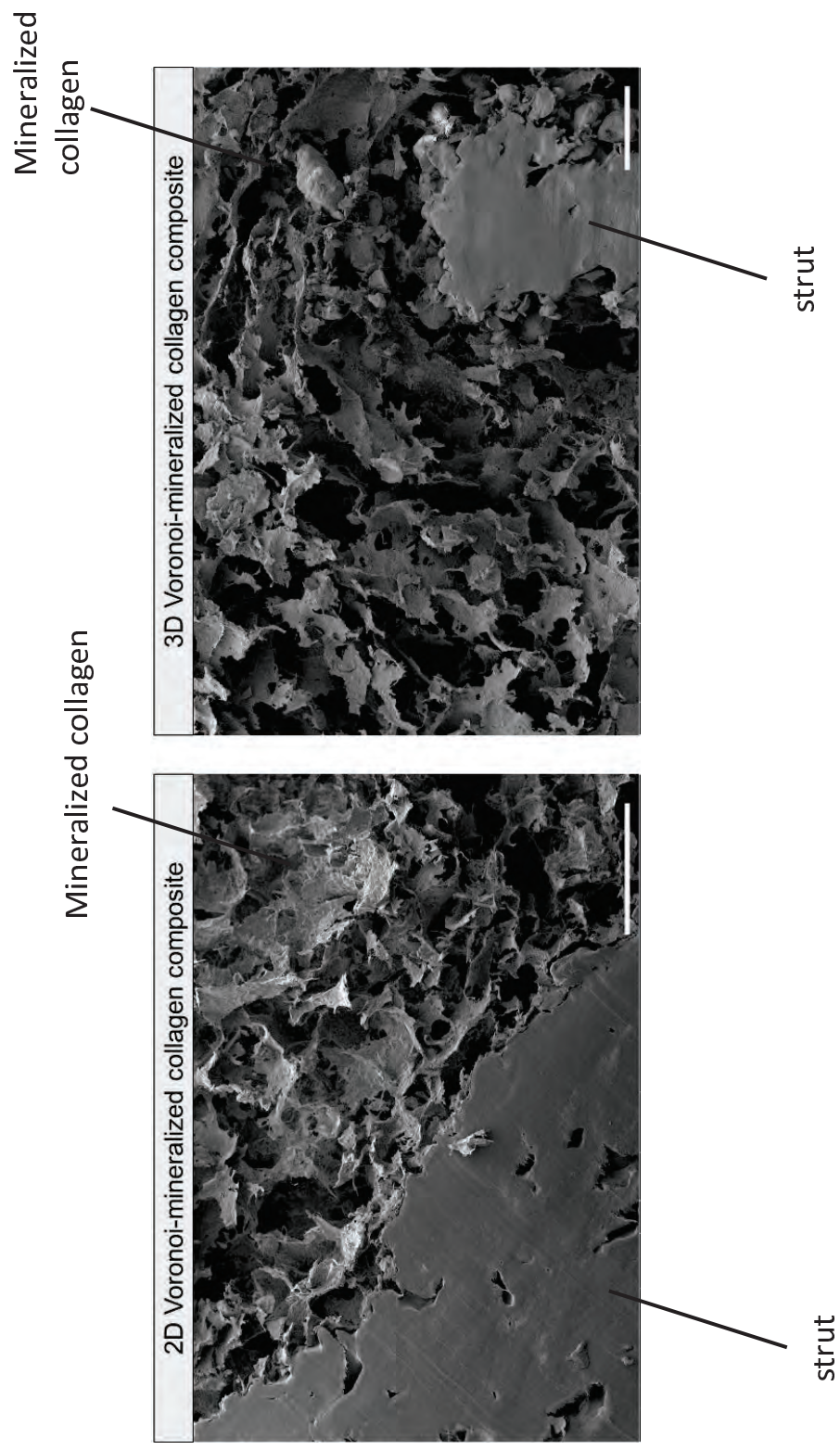


FIG. 14A

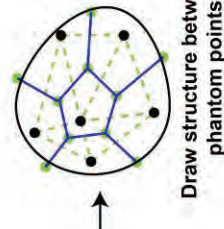
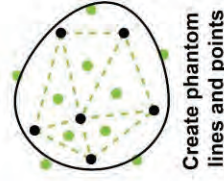
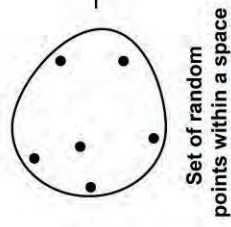


FIG. 14B

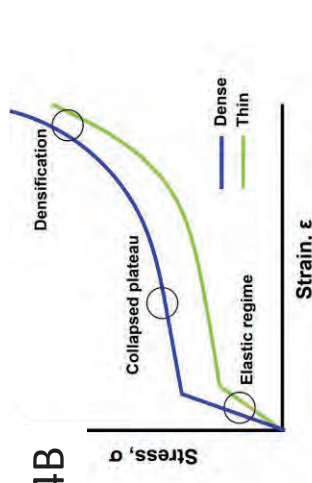
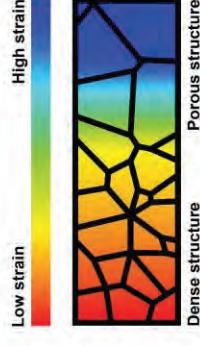
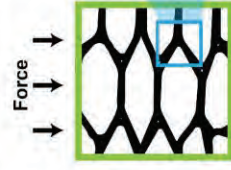
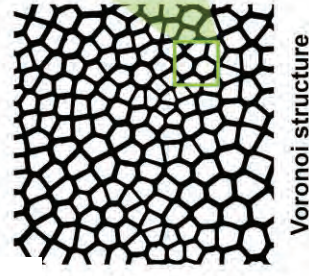


FIG. 14C



Strain localization via design

FIG. 14D

