BIOMIMETIC, MUSSEL-INSPIRED, BIOACTIVE BONE GRAFT SUBSTITUTE MATERIALS COMPRISING EXTRACELLULAR MATRICES: NOVEL COMPOSITIONS AND METHODS FOR BONE GRAFTS AND FUSIONS

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Abstract

Bone grafting is a surgical procedure used to create new bone. New bone is often needed in a broad range of healthcare applications, from spinal fusion to dental surgery. The gold standard bone graft is autologous bone, which is bone that is taken from and used in the same individual. However, autologous bone is limited in supply and is not always effective – and using it often requires an additional surgery for obtaining the donor bone and may cause morbidity (e.g., pain) at the site of obtainment. *Bone graft substitutes* are alternatives to autologous bone and aim to reduce or replace the need for autologous bone in bone grafting procedures. Current bone graft substitutes (e.g., Infuse™ Bone Graft, Medtronic; Memphis, TN) offer no better grafting outcomes to autologous bone, and in some cases carry significant complication profiles.

The present dissertation pertains to the creation and evaluation of novel bone graft substitute materials. These materials were designed to overcome the limitations of current bone graft substitutes, like bio-disparate designs and non-controlled growth factor delivery. At their core, the materials are porous, homogenously dispersed solid mixtures of pro-regenerative extracellular matrices (e.g., small intestinal submucosa) and inorganic components (e.g., calcium phosphates, bioactive glasses) in a compositional makeup that is biomimetic to bone. The materials are created from a "one-pot" liquid hydrogel solution at room temperature and physiologic pH, which allows virtually any additive (e.g., pharmaceuticals, minerals, cells) to be added during the synthesis and homogenously incorporated into the final material. In some embodiments, the materials are infused with polydopamine, conferring controlled growth factor delivery.

The materials underwent preclinical evaluation of bone forming efficacy and safety using a clinically translatable rat model of spinal fusion. In this model, use of one embodiment (termed "BioMim-PDA") achieved superior bone volume and quality at a 10-fold lower dose of recombinant human bone morphogenetic protein-2 relative to use of the Infuse™ Bone Graft; the overall fusion rate using BioMim-PDA was 97% (29/30 animals). In a separate study, a small molecule-loaded embodiment of the materials yielded a spinal fusion rate of 100% (16/16 animals). These results compare favorably to previous work, in which a recent meta-analysis of the rat model of spinal fusion reported an overall fusion rate of 42.5% using autologous (iliac crest) bone graft.

The ability to create a virtually infinite array of efficacious and scalable bone graft substitutes, as described herein, is unique and offers significant research and clinical value. Future work will focus on identifying graft compositions with the greatest therapeutic potential for bone grafting. The ultimate goal of this work is to create bone graft substitute materials that deliver reliable, safe, and effective grafting outcomes for patients requiring a bone graft.

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graft was allowed to stand for 15 minutes before implantation; the wetted grafts assumed nearly identical physical dimensions (lower middle and lower right) and handleability.

3.5 Grafts were evaluated using a clinically translatable rat model of spinal fusion. The L4-L5 transverse processes were exposed bilaterally and decorticated. rhBMP-2-adsorbed collagen sponge (left) or BioMim-PDA (right) was then implanted over the decorticated transverse processes bilaterally; both graft materials were easily moldable about the spine while retaining structural integrity.

3.6 Comparative histological staining images of the collagen sponge (left) and BioMim-PDA (right) are shown. Both the collagen sponge and BioMim-PDA are porous, acellular, and comprised of homogeneously dispersed collagens (H&E and Masson's trichrome stains). However, in contrast to the collagen sponge, BioMim-PDA is also comprised of homogeneously dispersed calcium phosphates (von Kossa stains), which confer osteoconductivity and osteoinductivity.

3.7 As shown via scanning electron microscopy, both the collagen sponge (left) and BioMim-PDA (right) are comprised of a porous, homogenous, fibrillar architecture. Magnification: 100× magnification; scale bar: 500μm.

3.8 Representative μCT slices and corresponding H&E histological images at the same level in the coronal plane are shown. An example of a unilateral fibrous nonunion (right side) following use of the collagen sponge and 0.2μg rhBMP-2 is shown. Spinal fusions – defined as continuous bridging bone from L4 to L5, bilaterally – are shown for the remaining groups.

Chapter 1

Introduction & Background

Bone grafting is a surgical procedure used to create new bone [1]. New bone is often needed in medicine and in dentistry [2]. For example, when a fracture is not healing, or when insufficient bone is present for dental implants, health care providers may perform a bone grafting procedure to create bone to treat the patient. In addition, bone grafting is commonly used in spinal surgery – specifically, in a procedure called spinal fusion or spinal arthrodesis [3]. In a spinal fusion procedure, bone grafting is used to create bone across a joint space to immobilize, or "fuse," segments of the spine. This is done to treat neck pain, back pain, and spinal instability either with or without nerve compression. More than 500,000 spinal fusion procedures are performed each year in the United States[4].

A bone graft is the material used in a bone grafting or arthrodesis/fusion procedure. Currently, the "gold standard" bone graft is autologous bone, which is bone that is taken from and used in the same individual being treated [5]. In this way, bone is moved from one location to another in the body. Because this graft is bone, it readily forms bone in a new location. However, autologous bone is limited in supply – and using autologous bone often requires an additional surgery for obtaining the donor bone and may cause morbidity (e.g., pain/fracture) at the site of obtainment [6]. For example, in spinal fusion surgery, autologous bone is often obtained from the bony pelvis or a rib for

bone grafting purposes. This generally lengthens the time of surgery and causes additional pain to the patient.

Alternatives to autologous bone grafts comprise grafts that aim to reduce or replace the need for autologous bone in bone grafting procedures, while effectively and safely creating new bone [7]. Autologous bone graft alternatives are either allograft bone or bone graft substitutes. Allograft bone is bone that is harvested from human cadavers, sterilely processed and transplanted into a recipient. Bone graft substitutes are all other bone graft materials, including derivatives of allograft bone (e.g., "demineralized bone matrix") and laboratory-created materials [8, 9]. In general, allograft bone is less efficacious than autologous bone at creating new bone and is associated with a risk of immunogenic rejection and disease transmission. Bone graft substitutes, in contrast, offer expanded opportunities to create novel bone graft materials.

An example of a leading bone graft substitute is the Infuse™ Bone Graft, which is marketed by Medtronic [10]. This bone graft substitute comprises two parts: (1) a Type I collagen sponge, derived from bovine Achilles tendon, and (2) a solution of recombinant human bone morphogenetic protein-2 (rhBMP-2). To use, the collagen sponge is soaked in the solution of rhBMP-2 for a minimum of 15 minutes, and the wetted collagen sponge is then implanted for bone grafting. The Infuse™ Bone Graft typically results in similar rates of bony arthrodesis (e.g., spinal fusion) compared to autograft bone [11]. However, this product is associated with relatively frequent adverse events from the rhBMP-2 dosage, including life-threatening complications, which led the United States Food and Drug Administration to issue a Public Health Notification about rhBMP-2 use in 2008 [12]. The potentially life-threatening complications associated with rhBMP-2 use in

Infuse™ are believed to arise from the local bolus delivery of rhBMP-2, which may exceed one-million-times the normal physiological concentrations [13, 14].

Another example of a bone graft substitute is Vitoss (Stryker), which is the #1 selling synthetic bone graft, with over 600,000 implantations worldwide [15]. This bone graft is comprised solely of beta-tricalcium phosphate, which is similar to the primary mineral component of bone (i.e., hydroxyapatite). Vitoss is created with an interconnected porous structure resembling human cancellous bone. Because this bone graft substitute is comprised solely of calcium phosphate, it is brittle and, if used improperly, is susceptible to breakage during implantation. For spinal fusion, a more flexible bone graft that can be molded about the spine while holding together is desirable.

The extracellular matrix (ECM) is the physical microenvironment in which cells exist [16]. It provides a substrate for cell anchorage, serves as a tissue scaffold, guides cell migration during embryonic development and wound repair, and plays key roles in tissue morphogenesis. In addition, the ECM is responsible for transmitting environmental signals to cells, which ultimately affects cell proliferation, differentiation, and death [17].

In general, the ECM is comprised of four major categories of biomolecules: (1) structural proteins (e.g., collagen and elastin), which in part provide strength and resilience; (2) glycosaminoglycans and proteoglycans (e.g., hyaluronate, chondroitin sulfate, and heparin sulfate), which in part cushion cells and sequester physiologically important proteins; (3) glycoproteins and other matricellular proteins (e.g., fibronectin, laminin, and osteopontin), which in part aid in cell adhesion, migration, growth, and differentiation; and (4) growth factors (e.g., fibroblast growth factors, transforming growth factor beta, and vascular endothelial growth factor), which regulate diverse

cellular processes. Different combinations of these molecules tailor the matrix for different functions depending on the physiological needs and adaptations of the tissue. Further, ECMs may be isolated and used for clinical indications.

ECMs are commercially available for soft-tissue grafts, including ACell's MatriStem Urinary Bladder Matrix™ (UBM) for wound care and Cook Medical's Biodesign® Small Intestinal Submucosa (SIS) for a wide-range of general and reconstructive surgical indications [18]. Both of these products are derived from porcine tissue (i.e., bladder or small intestines). Further, both are marketed to facilitate the restoration, or regeneration, of site-appropriate functional tissue because of the unique characteristics of the ECM, which allow for intimate cell contact and new tissue ingrowth. For example, because of their unique characteristics, ECMs may promote a pro-regenerative (e.g., "macrophage M2-phenotype") remodeling healing response rather than a pro-inflammatory (e.g., "macrophage M1-phenotype") healing response seen with other scaffolds [19]. The MatriStem UBM products include an intact epithelial basement membrane on one surface and a lamina propria layer on the opposite surface; in contrast, the Biodesign® SIS product is comprised solely of the submucosal layer of proximal jejunum. Both products are processed from raw materials via mechanical and chemical means that minimize the loss of natural ECM components and structure, while yielding decellularized and sterile tissue. When implanted, these products are believed to be gradually remodeled in a way that helps the body restore itself.

MastriStem® products are available as sheets (e.g., MatriStem Wound Matrix, MatriStem Multilayer Wound Matrix, and MatriStem Burn Matrix) and particles (e.g., MatriStem MicroMatrix) with a particle size <500 micrometers. In contrast, the

Biodesign® products are available as sheets (e.g., Biodesign Dural Graft, Biodesign Hernia Graft, and Biodesign Otologic Repair Graft), combination strip products (e.g., Biodesign Staple Line Reinforcement), plugs (e.g., Biodesign Fistula Plug Set), and cylinders (e.g., Biodesign Nipple Reconstructin Cylinder). Cook Medical separately offers a product called Powder Extracellular Matrix, which is micronized particles of porcine-derived SIS. CorMatrix® is another company that creates porcine-derived SIS sheets (e.g., CorMatrix ECM for Vascular Repair). All of these ECM products are initially created as sheets; the ECM is a layer, or sheet, isolated from the host tissue. Powdered versions may then be created from these sheets or sheet pieces (e.g., by cryogenically milling the sheets). Further, the indications for all of these products are for soft tissues; none are indicated for creating bone (e.g., as a bone graft). That is, there are no commercially available bone grafts involving pro-regenerative ECMs like UBM or SIS.

Chapter 2

Biomimetic, Mussel-inspired, Bioactive Bone Graft Substitute Materials Comprising ECM

2.1 Summary

This dissertation pertains to novel bone graft substitute materials that are porous, homogenously dispersed solid mixtures of pro-regenerative extracellular matrix (ECM) particles and inorganic components (e.g., calcium phosphates, bioactive glasses) – and potentially any additional homogenously incorporated small molecule (e.g., drug) and/or mineral. That is, a bone graft material of the present dissertation is created from a "one-pot" liquid ECM hydrogel solution at room temperature and physiologic pH, which allows virtually any bioactive small molecule and/or mineral to be added to the bone graft substitute during the synthesis. In some embodiments, the materials are activated with polydopamine, which confers controlled growth factor (e.g., rhBMP-2) delivery.

The present materials were designed and created to overcome the limitations of current leading bone graft substitutes. For example, primary limitations of the Infuse™ Bone Graft include its bio-disparate design and supraphysiologic burst release of growth factor (i.e., rhBMP-2). To solve this, the materials of the present dissertation involve a biomimetic scaffold, comprised of both inorganic (e.g., calcium phosphate) and organic (i.e., ECM) components, similar to the composition of bone. This is in contrast to the Type I collagen sponge used in the Infuse™ sponge. Further, the addition of

polydopamine confers a controlled and sustained growth factor release, which serves to overcome the bolus growth factor delivery method that complicates the existing Infuse™ product. In addition, the inventive biomimetic design confers flexibility to the graft, which serves to overcome the brittleness of the Vitoss product.

Further, these are the first biomimetic bone graft compositions that utilize ECM, which is a superior organic component source compared to Type I collagen alone. Finally, the synthetic strategy described herein enables the creation of bone graft substitutes with a wide array of homogeneously dispersed known additives, including small molecules and minerals, since the grafts are created from a "one-pot" liquid ECM hydrogel solution at room temperature and physiologic pH. For example, antibiotics may be added to the ECM hydrogel solution during the synthetic process to yield a bone graft material with homogeneously dispersed antibiotics. This bone graft substitute may decrease the risk of infection while effectively creating new bone or bone fusions. The capacity to create this large array of non-autologous bone graft substitutes is unique to the present work and offers significant research and clinical value.

In accordance with a first embodiment, the present work provides a biomimetic bone graft material comprising one or more inorganic particles (e.g., calcium phosphates, bioactive glasses), ECM particles comprising one or more biomolecules including collagens, elastins, glycosaminoglycans, proteoglycans, glycoproteins, matricellular proteins, and a combination thereof in a mixture. In accordance with a second embodiment, the present work provides a biomimetic bone graft material comprising one or more calcium phosphate particles, ECM particles comprising one or more biomolecules including collagens, elastins, glycosaminoglycans, proteoglycans,

glycoproteins, matricellular proteins, and a combination thereof, and further comprising one or more additional biologically active agents, in a mixture.

A third embodiment provides a method of making a bone graft material comprising the steps of: a) digesting ECM particles comprising structural proteins, glycosaminoglycans, proteoglycans, glycoproteins, matricellular proteins, growth factors, and a combination thereof, with a protease; b) neutralizing the digested ECM particles and forming a mixture comprising functional proteins or functional portions thereof; c) adding inorganic particles (e.g., calcium phosphates, bioactive glasses) to the mixture of c) and forming a homogeneously dispersed mixture; d) pouring the homogeneously dispersed mixture into a mold; e) incubating the mixture until it solidifies; f) lyophilizing the mixture forming a material; g) contacting the material with a solution of dopamine forming an activated material infused with polydopamine; and h) lyophilizing the activated material forming a bone graft material comprising an EC.

In accordance with a fourth embodiment, the present work provides the method above further comprising the step of contacting the bone graft material comprising an ECM with a solution containing a growth factor. In accordance with a fifth embodiment, the present work provides the method above further comprising the step of lyophilizing the bone graft material. A sixth embodiment provides the method above further comprising the step of adding ECM particles before pouring the homogenously dispersed mixture into a mold.

2.2 Detailed Descriptions

Suitable tissue used in the present dissertation for making ECM includes small intestine tissue, bladder tissue, adipose tissue, stomach tissue, large intestine tissue, bone tissue, brain tissue, cartilage tissue, heart tissue, kidney tissue, liver tissue, pancreas tissue, trachea tissue, lung tissue, skeletal muscle tissue, pharynx tissue, esophagus tissue, spleen tissue, skin tissue, and/or tendon tissue. The tissue may come from most animals including pig. The tissue is used shortly after it is removed from the animal and is raw tissue, for example. The methods of the present dissertation may include additional steps such as sieving the ECM particles to a desired size.

Typically, the ECM particle composition comprises collagen, elastin, glycosaminoglycan, proteoglycan, glycoprotein, matricellular protein, various growth factors, and combinations thereof. In some embodiments, the ECM particle compositions have particles less than 700 μm in diameter and may be dry, for example.

The glycosaminoglycan in the ECM particle compositions may be a hyaluronate, heparin, chondroitin sulfate, heparin sulfate, dermatan sulfate, keratan sulfate, and a combination thereof. The proteoglycan in the ECM particle compositions may be an aggrecan, perlecan, brevican, decorin, lumican, neurocan, versican, agrin, Type XVIII collagen, leprecan, proteoglycan 2, proteoglycan 3, hyaluronan and proteoglycan link protein, osteoadherin, prolargin, epiphycan, osteoglycin, chondroadherin, chondroadherin-like protein, nephrocan, podocan, podocan-like protein, testican, lubricin, endocan, and a combination thereof. The glycoprotein in the ECM particle compositions may be a fibronectin, laminin, biglycan, entactin, dermatopontin, colligin, nidogen,

asporin, emilin, fibrillin, bone sialoprotein, matrilin, microfibrillar-associated protein, multimerin, nephronectin, osteonectin, SPARC-like protein, insulin-like growth-factor-binding protein, kielin/chordin-like protein, and a combination thereof. The matricellular protein in the ECM particle compositions may be a tenascin, thrombospondin, osteopontin, CCN family protein, fibulin, periostin, galectin, fibrinogen, vitronectin, ameloblastin, osteocalcin, cartilage intermediate-layer protein, dentin matrix acidic phosphoprotein, dentin sialophosphoprotein, matrix gla protein, latent transforming growth-factor beta-binding protein, or a combination thereof. The growth factors of the ECM particle composition may be a fibroblast growth factor, transforming growth factor, vascular endothelial growth factor, insulin-like growth factor, hepatocyte growth factor, epidermal growth factor, platelet derived growth factor, neurotrophin, erythropoietin, bone morphogenetic protein, interleukin, colony-stimulating factor, angiopoietin, or a combination thereof.

One embodiment of the present dissertation is a method of making a bone graft material comprising the ECM particle composition. The method comprises the steps of: a) digesting ECM particles comprising structural proteins, glycosaminoglycans, proteoglycans, glycoproteins, matricellular proteins, growth factors, and combinations with a protease; b) neutralizing the digested ECM particles forming a mixture comprising functional proteins; c) adding inorganic components (e.g., calcium phosphates, bioactive glasses) to the mixture forming a homogeneously dispersed mixture; d) pouring the homogeneously dispersed mixture into a mold; e) incubating the mixture until it solidifies. In some embodiments the method further comprises: f) lyophilizing the mixture forming a material; g) contacting the material with a solution of dopamine

forming an activated material infused with polydopamine; and h) lyophilizing the activated material forming a bone graft material comprising an ECM.

Other embodiments of the present dissertation comprise the step of contacting the bone graft material comprising an ECM with a solution containing a growth factor. The material at that point can be used as is, or it can then be lyophilized. Suitable growth factors used in the present invention include BMPs, calcitonin, parathyroid hormone, AB204, angiopoietin, erythropoietin, fibroblast growth factors, transforming growth factors, insulin, NEL-like protein 1, peptide B2A, insulin-like growth factor, vascular endothelial growth factor, platelet derived growth factor, hepatocyte growth factor, epidermal growth factor, interleukins, colony stimulating factors, neurotrophins, or combinations thereof. Additional growth factors used in the present work include BMP-2, VEGF-165, PDGF-BB, and combinations thereof.

Other embodiments of the present work include a step of adding a pharmaceutical agent or mineral before pouring the homogeneously dispersed mixture into a mold. Another embodiment includes a step of adding ECM particles before pouring the homogenously dispersed mixture into a mold. Examples of proteases that may be used include a pepsin, chymotrypsin, matrix metalloproteinase, collagenase, alcalase, papain, cathepsin, trypsin, and a combination thereof. In some embodiments, the pepsin used is derived from porcine gastric mucosa. Any suitable shape or size of mold may be used. In an embodiment the mold used may have the dimensions of about 3 inches by about 2 inches though a variety of molds may be use that facilitate the attachment of a bone graft material to a bone, a broken bone, or a bony defect.

An example of a dilute base used in the present work is sodium hydroxide and the solution has a concentration of about 5 to 25 mg ECM/mL, 5 to 15 mg ECM/mL, or 10 mg ECM/mL. Suitable incubating temperatures include from 20 to 50 °C, 30 to 40 °C, or 37°C until the mixture solidifies. In some embodiments, the bone graft material is substantially free of water or dry. In some embodiments, the inorganic particles have a size less than 200 μm, 150 μm, 100 μm, 90 μm, 80 μm, 70 μm, 60 μm, 50 μm, 40 μm, 30 μm, 20 μm, or 10 μm. The inorganic particles may comprise a combination of particles (e.g., the calcium phosphates beta-tricalcium phosphate and hydroxyapatite). In some embodiments, the bone graft material is in the range from 1 part ECM to 1 part inorganic component to 1 part ECM to 15 parts inorganic component. In some embodiments, the digestion of ECM particles occurs using 10 mg/ml of the ECM particle, 1 mg/mL of pepsin, and 0.01N HCl for between about 6 hours to about 72 hours. In an embodiment, the digestions occurs in 24 hours.

Another embodiment of the present work is a bone graft material comprising one or more inorganic particles (calcium phosphates, bioactive glasses); and ECM particles comprising one or more of collagens, elastins, glycosaminoglycans, proteoglycans, glycoproteins, matricellular proteins, growth factors and a combination thereof. Another embodiment of the present dissertation is a bone graft material comprising inorganic particles (calcium phosphates, bioactive glasses) having a size less than 200 μm homogenously distributed in a matrix of ECM composition comprising one or more of the following: collagens, elastins, glycosaminoglycans, proteoglycans, glycoproteins, matricellular proteins, growth factors and combinations thereof.

In accordance with another embodiment, the present dissertation provides the bone graft material further comprising one or more of the following: pharmaceutical agents; minerals; polydopamine; growth factors, such as rhBMP-2; small molecules, such as oxysterols; and a combinations thereof.

In accordance with another embodiment, the present work provides methods of bone grafting. These methods include the steps of implanting any one of the bone graft materials of the present work in a subject having a medical condition requiring a bone graft at the site of the medical condition; and creating new bone growth *in vivo*. The methods of the present invention may be used to treat medical conditions including fractures; knee injuries; hip injuries; missing teeth replacement, tooth implants requiring bone for a dental implant; treatment of critical-sized bony defects; bone injuries or defects requiring a fusion procedure including, for example, foot, ankle, fingers, wrists, spinal fusion; or combinations thereof.

2.3 Examples

2.3.1 Extracellular Matrix (ECM) Particles

Extracellular Matrix (ECM) particles are used in the present work. ECM particles may be commercially purchased (e.g., Powder Extracellular Matrix [Cook Biotech] and MicroMatrix® [ACell]), produced from commercially purchased non-particulate ECM products (e.g., MatriStem Wound Matrix, Biodesign Dural Graft, and CorMatrix® ECM for Vascular Repair), or produced using the methods described herein. One embodiment of making ECM particles includes obtaining fresh porcine tissue (e.g., bladder and small intestines) from a slaughterhouse within 30 minutes of death of the animal. A

"slaughterhouse inspector" from the United States Department of Agriculture oversees the obtainment of healthy, fresh tissue. Tissue from young market pigs (e.g., less than or equal to 300 lbs. in weight) of any breed may be used. The tissue is placed into a seal tight plastic bag, cooled to approximately 32 °C, and then transported to the laboratory (or similar facility) for processing.

At the laboratory, waste (e.g., urine and chyme) is removed from the tissue with running water. The tissue may be cut open and/or into smaller pieces to facilitate this process. For example, small intestines may be cut into approximately 12-inch-long pieces and each piece then cut open lengthways, such that the mucosa layer is visualized on one side and the serosa layer is visualized on the other side. Waste (chyme) may then be easily removed by pulling the tissue between a tight space created between one's fingers under running water. The tissue is rinsed with distilled water at room temperature, and then placed into a container with distilled water cooled to approximately 0 °C while the rest of the tissue(s) is similarly cleaned.

Of note, only proximal jejunum is used to create small intestine submucosa (SIS); the rest of the small intestine (e.g., duodenum and ileum) is not used. Practically, proximal jejunum is inferred to start 18 inches from the pyloric sphincter and include approximately 6 feet of tissue thereafter, or 1 foot proximal to the first appearance of Peyer's patches. The presence of Peyer's patches, or small circular patches of roughened areas in the tissue, indicates ileum tissue and should not be used in the making of SIS. Excess water is removed from the cleaned tissue by pulling the tissue between a tight space created between one's fingers. The tissue is then placed into a seal-tight plastic bag and frozen at -20 °C for between 12 and 96 hours.

The tissue is thawed in the sealed plastic bag under running tap water and then kept at approximately 0 °C. The ECM layer is then obtained on a disinfected laboratory (or similar facility) bench by hand using: forceps, scissors, a scalpel, gauzes, and/or thin pieces of metal or plastic for scraping (e.g., polystyrene lids from 96 well-plates). For example, for SIS, a 12-inch-long piece of proximal jejunum, cut open lengthways so as to expose the mucosa and serosa layers, is laid down flat (mucosa layer facing up) on the bench. The mucosa layer is then scraped away using the edges of two polystyrene lids from 96 well-plates pulling in opposite directions. The tissue is then flipped over (serosa side facing up) on the bench, and the serosa layer is similarly removed. Forceps, scissors, a scalpel and/or gauzes may be used to facilitate this process (e.g., by creating an initial small cut in the serosa layer, from which the serosa layer may be scraped away).

Examples of the final ECM at this stage are shown in Figs. 1 and 2 for SIS and UBM, respectively. The ECM is then cut into small pieces (approximately 1” x 1”, Length x Width) using scissors to facilitate the chemical processing step (i.e., increase surface area and prevent clumping of ECM pieces, which facilitate decellularization and sterilization), which follows. The ECM pieces are temporally stored in a beaker containing distilled water at approximately 0 degrees Celsius until all ECM pieces from the available tissue have been generated.

The ECM is chemically processed to effectively decellularize and sterilize the material using a “decellularizing and sterilizing solution (DSS).” The DSS, used at an approximate concentration of 0.2 g ECM per mL DSS, is comprised of 95.67% distilled water, 4% absolute (200 proof) alcohol, and 0.33% peracetic acid (~30% in dilute acetic acid). An approximate concentration is used because the mass of ECM is not precisely

known because water is present in the ECM (i.e., excess water is removed from the ECM before weighing by pulling the pieces between a tight space created between one's fingers; however, the ECM is not fully dried at the time of weighing).

Table 2.1 provides guidance for making the DSS [20]. This method may be scaled proportionately. An appropriately sized Erlenmeyer flask should be used for chemical processing based on the amount of ECM being processed and the total volume of DSS required. For example, for 60 grams of ECM, which corresponds to 1200 mL of DSS, a 2 L Erlenmeyer flask would be appropriate.

Table 2.1: Volumes of chemical reagents required for making the "decellularizing and sterilizing solution (DSS)" based on the amount of ECM added to an appropriately-sized Erlenmeyer flask for chemical processing.

Added ECM (g, approximate)	**Added Volume (mL)**			
	Distilled Water	**Absolute Ethanol (200 proof)**	**Peracetic Acid (~30% wt. % in dilute acetic acid)**	**Total**
20	383	16	1.33	400
40	765	32	2.67	800
60	1148	48	4.00	1200
80	1531	64	5.33	1600

The weighted amount of ECM pieces are added to an appropriately-sized Erlenmeyer flask (chart above) and the required volume of DSS is then added to the flask. The flask is then sealed with Parafilm or similar means (Fig. 3). The flask containing the ECM and DSS is then shaken for 2 h at 200 RPM using an orbital shaker.

From here on, all work is performed in a way that maintains sterility of the ECM. The liquid is removed from the flask and replaced with the same volume of phosphate buffered saline (PBS). The flask is then resealed with Parafilm or similar and shaken on an orbital shaker for 15 min at 200 RPM. The liquid is then removed from the flask and replaced with the same volume of water. The flask is then resealed with Parafilm or similar and shaken on an orbital shaker for 15 min at 200 RPM. The liquid is then removed from the flask and replaced with the same volume of water. The flask is then resealed with Parafilm or similar and shaken on an orbital shaker for 15 min at 200 RPM. The liquid is then removed from the flask and replaced with the same volume of PBS. The flask is then resealed with Parafilm or similar and shaken on an orbital shaker for 15 min at 200 RPM. The liquid is then removed from the flask and replaced with the same volume of water. The flask is then resealed with Parafilm or similar and shaken on an orbital shaker for 15 min at 200 RPM.

The pH of the liquid is then confirmed to be neutral. If the pH is confirmed to be neutral, then the liquid is removed and the ECM pieces are transferred to one or more 50 mL conical tubes for lyophilization. The pieces are placed lengthways along the bottom 1/3 of the horizontally-held conical tube to maximize surface area for lyophilization. If the pH is not neutral (e.g., acidic), then the liquid is removed from the flask and replaced with the same volume of PBS. The flask is then resealed with Parafilm or similar and shaken on an orbital shaker for 15 min at 200 RPM. The liquid is then removed from the flask and replaced with the same volume of water. The flask is then resealed with Parafilm or similar and shaken on an orbital shaker for 15 min at 200 RPM. The pH of the liquid is then confirmed to be neutral, whereby, upon confirmation of neutral pH, the

above-described steps regarding lyophilization follow. If the pH is still not neutral, the ECM pieces may be further washed with PBS followed by water, as described above, until neutral pH is obtained.

The tube containing the ECM is then laid horizontally in a -20 °C freezer for between 12 and 96 hours. The frozen ECM (-20 °C) is then lyophilized (vacuum approximately 0.1 mbar and the collector temperature is approximately -60 °C) for between 18 and 48 hours. Examples of final materials at this stage are illustrated for SIS and UBM in Fig. 4.

The lyophilized ECM is cut with scissors into small pieces (approximately 0.1 mm^3) and then added to polycarbonate vials (e.g., Poly-Vial Set, SPEX SamplePrep) for cryogenic milling. The vials are filled approximately 1/3 full with ECM pieces. The ECM is then cryogenically milled in liquid nitrogen (e.g., SPEX SamplePrep, 6870 Freezer/Mill) using the following parameters: Cycles: 7, Pre-cool: 10 min, Run time: 2 min, Cool time: 3 min, Rate: 10 cycles/sec.

The cryogenically milled ECM is sieved through a mesh sieve (e.g., No. 40 mesh; 425-micrometers) (Fig. 5). The sieved ECM particles are then transferred to a 50-mL conical tube and stored at -80 o C until needed (for up to 1 year). The ECM particles may be characterized by a variety of means, including scanning electron microscopy (e.g. microarchitecture) and proteomics analysis. The composition of the ECM particles varies based on the source tissue.

As an example, UBM particles are comprised of more than eighty unique ECM molecules. This is in contrast to other bone graft substitute materials that comprise only

one type of organic molecule (e.g., Type I collagen in the Infuse™ Bone Graft). The vast majority of the ECM molecules in UBM are collagens (approximately 97% by dry weight of UBM), including Types I, III, IV, V, VI, VII, VIII, IX, XI, XII, XIV, XV, XVIII, and XXI; the most abundant is Type I (approximately 65% of the total collagen content) followed by Type III (approximately 25% of the total collagen content). Additionally, proteoglycans and glycoproteins each constitute approximately 1% of the ECM molecules in UBM, whereas glycosaminoglycans, matricellular proteins, and growth factors together comprise the remaining approximately 1%. The most abundant of the proteoglycans, by dry weight of UBM, is decorin (approximately 0.3%) followed by lumican (approximately 0.2%). The most abundant of the glycoproteins, by dry weight of UBM, are dermatopontin and microfibrillar associated protein 2 (each approximately 0.1%). Additionally, by dry weight of UBM, elastin constitutes approximately 0.05% of the ECM molecules in UBM. The most abundant matricellular proteins, by dry weight of UBM, are fibrinogen (approximately 0.1%) and fibulin (approximately 0.03%). Additionally, the most abundant growth factor, by dry weight of UBM, is transforming growth factor-beta (approximately 0.04%). It is believed herein that the biomimetic combination of biologically active molecules in ECMs will confer enhanced regenerative potential – and bone formation – to bone graft substitute materials, as described in the present invention, relative to the Type I collagen alone which is currently used in some commercially available bone graft substitute materials.

2.3.2 Method of Making SIS Particles

Fresh, healthy porcine small intestines were obtained from Wagner Meats, LLC, Mt. Airy, MD within 30 minutes of slaughtering a young market Yorkshire hog

(approximately 250 lbs. in weight). The small intestines were obtained with the assistance of a “slaughterhouse inspector” from the United States Department of Agriculture. The distal part of the stomach, including the pyloric sphincter, was also obtained for orientation purposes. The tissue was placed into a seal-tight, plastic bag and transferred to an insulated container filled with cold packs. The tissue was then transferred to the laboratory (Johns Hopkins Hospital, Baltimore, MD).

The tissue was laid out on a disinfected laboratory bench. The pyloric sphincter was identified, and the first 18 inches of small intestines (distal to the pyloric sphincter; corresponding to the duodenum) was cut and removed from the rest of the small intestines. Next, a 12-inch-long piece of proximal jejunum was cut off, and the tissue then cut open lengthways. The chyme was removed from the tissue by pulling the tissue through a tight space created between my fingers under running water. The cleaned piece of small intestines was rinsed in distilled water and then temporarily stored in a beaker of distilled water cooled on ice. The next 12-inches of jejunum were cut off, cleaned and stored similarly. This process was repeated until the appearance of Peyer’s patches, which signaled the end of the jejunum and beginning of the ileum. Any jejunum within 1-foot of the first appearance of Peyer’s patches was identified and discarded. The rest of the jejunum was kept for the creation of SIS. Excess water from the cleaned and collected proximal jejunum was removed by pulling the pieces of tissue through a tight opening between my fingers. The pieces were then transferred to a seal-tight plastic bag and frozen for 36 hours at -20 °C.

The proximal jejunum was then mechanically processed to obtain SIS. First, the laboratory bench was disinfected, and the following tools were prepared: scissors,

forceps, scalpel, gauzes, and four polystyrene lids from 96-well plates. The frozen proximal jejunum was thawed in the sealed bag under running water, and, while still in the sealed bag, then placed on ice. A piece of proximal jejunum was obtained from the bag and laid mucosa-side up on the laboratory bench. The mucosa layer was scraped away by applying the edges of the polystyrene lids to the tissue in an opposite-direction pulling motion. Next, the tissue was flipped over (serosa-side up), and the muscularis and serosa layers were similarly removed using the polystyrene lids. The tissue was scraped with sufficient force to remove the mucosa and muscularis/serosa layers, but not too much that would tear the submucosa layer. A beautiful white, near-translucent material with cobweb-like connecting lines was obtained. To maximize subsequent decellularization and sterilization, this material was cut into small pieces (approximately 1" x 1", Length x Width), which were then transferred to a beaker containing distilled water, on ice. The process was then repeated for the remaining pieces of proximal jejunum.

The small pieces of SIS were then effectively decellularized and sterilized. First, excess water was removed from the pieces by pulling the pieces through a tight space between one's fingers, and then all the pieces were weighed. The obtained weight was approximately 60 g. Accordingly, 1200 mL of "decellularization and sterilizing solution (DSS)" was made, comprising 1148 mL (95.67%) of distilled water, 48 mL (4%) of 200-proof ethanol, and 4 mL (0.33%) of peracetic acid (from 32 wt. % in dilute acetic acid). The peracetic acid, which is a corrosive substance, was handled in a fume hood. A 2-L Erlenmeyer flask, previously cleaned and autoclaved, was obtained. The ECM (60 g) was then added to the Erlenmeyer flask, followed by the 1200 mL of the DSS. The flask

containing the SIS and DSS was sealed with Parafilm, and then shaken for 2 h at 200 RPM and room temperature using an orbital shaker (VWR Symphony Model 5000I/R). Hereafter, effective sterility was maintained via tools and techniques.

The decellularized and sterilized SIS was then washed. First, the liquid was removed from the flask by pouring it off, and 1200 mL of PBS was then added to the flask. The flask was then re-sealed and then placed back onto the orbital shaker for 15 minutes at 200 RPM. The liquid was then removed, and 1200 mL of water was added to the flask. The flask was then re-sealed and then placed back onto the orbital shaker for 15 minutes at 200 RPM. The liquid was then removed, and 1200 mL of water was added to the flask. The flask was then re-sealed and then placed back onto the orbital shaker for 15 minutes at 200 RPM. The liquid was then removed, and 1200 mL of phosphate buffered saline was added to the flask. The flask was then re-sealed and then placed back onto the orbital shaker for 15 minutes at 200 RPM. The liquid was then removed, and 1200 mL of water was added to the flask. The flask was then re-sealed and then placed back onto the orbital shaker for 15 minutes at 200 RPM. The pH was confirmed at 6.9. The liquid was then removed, and the ECM pieces were transferred to two 50-mL conical tubes for lyophilization. The ECM pieces were placed lengthways along the bottom 1/3 of the horizontally-held conical tubes to maximize surface area for lyophilization.

The two conical tubes containing the SIS were then placed horizontally in a -20 degrees Celsius freezer for 18 hours. The frozen ECM (-20 °C) was then lyophilized (Labconco Freeze Dry System/Freezone 4.5; vacuum approximately 0.1 mbar; collector temperature approximately -60 °C) for 36 hours, yielding two soft white SIS materials in elongated semi-cylindrical shapes. The mass of SIS obtained was 2.5 g.

The lyophilized SIS was then cryogenically milled. First, the SIS was cut into small pieces of approximately 1 mm3 in a cell/tissue culture hood. The small pieces were then transferred into polycarbonate vials (Small Poly-Vial Set, SPEX SamplePrep), approximately 1/3 full and then cryogenically milled (SPEX SamplePrep, 6870 Freezer/Mill) using the following parameters:

i. Cycles: 7

ii. Pre-cool: 10 min

iii. Run time: 2 min

iv. Cool time: 3 min

v. Rate: 10 cycles/sec

The process was repeated until the entire SIS was milled. The powder was collected in a 50- mL conical tube. The cryogenically milled SIS was then sieved through a No. 40 (425-micrometer) mesh sieve, transferred to a 50-mL conical tube, and then stored at -80 °C. The mass of sieved SIS obtained was 2.2 g.

2.3.3 Biomimetic Bone Graft Substitute Material

Bone graft materials of the present dissertation are prepared with ECM particle compositions described above. One embodiment begins with digesting ECM particles with a protease in dilute acid at room temperature to create a digested ECM solution. For example, a protease (e.g., lyophilized pepsin from porcine gastric mucosa) is dissolved in a dilute acid (e.g., 0.1 N HCl) at a concentration of 1 mg protease/mL acid (e.g., 1 mg pepsin/mL 0.1 N HCl) in a sealed flask (e.g., beaker) while stirring at room temperature. The protease is fully dissolved when the solution is completely homogenous. Sieved

ECM particles (created per the above) are added to this solution at a concentration of 10 mg ECM/mL solution. Alternatively, the protease solution may be added to sieved ECM particles in a flask (e.g., beaker). The flask is sealed with Parafilm or similar, and the mixture is allowed to stir (via magnetic stir bar and stir plate) at room temperature for about 24 h. The ECM should be completely dissolved, yielding a homogenous mixture without identifiable ECM particles, after 24 h (Fig. 6). As a variant, the mixture may be stirred for less than 24 hours (e.g., 1 h, 2 h, 3 h, etc.), which results in ECM particles with more native structure and function in the final bone graft material (paragraphs below).

A dilute base (e.g., 0.1 N sodium hydroxide) is added dropwise to neutralize the digested ECM solution (approximately 1/10 the volume of acid described above) while stirring at room temperature. The pH is verified using pH strips, as the mixture at this point is too thick to use a liquid-based pH meter. Calcium phosphate particles, sieved through a mesh sieve (e.g., No. 325 mesh; 44-micrometers), are added to the mixture while stirring at a ratio from 1:1 to 1:20 (ECM:calcium phosphate) (Fig. 7). The mixture is allowed to stir for approximately 2 minutes to ensure homogenous mixing of the ECM and calcium phosphate particles; after 2 minutes, homogenous mixing is achieved (Fig. 8). As a possible variant, a buffer (e.g., 10 x PBS) may be added to the mixture before neutralizing with dilute base to help ensure a neutral pH and/or dilute the mixture to aid in the subsequent homogenous mixing of the ECM and calcium phosphate particles. However, diluting the mixture too much (e.g., < 7 mg ECM/mL solution) may cause the calcium phosphate particles to settle during the subsequent steps (paragraph below), resulting in poorly (i.e., not homogeneously) mixed ECM and calcium phosphate particles; this is to be avoided. Another possible variant is the addition of any powdered,

granulized, liquid, and/or gas formulation of a pharmaceutical (e.g., drug) and/or mineral to the neutralized ECM solution in addition to or in place of the calcium phosphate particles, for similar homogeneous incorporation in the bone graft material by stirring. This ability to homogeneously incorporate any such additive into a bone graft material at room temperature and neutral pH is a unique characteristic of the present work and offers significant research and clinical value. As examples, the pharmaceutical/mineral may include: antibiotics, growth factors, cytokines and chemokines, small molecules, carbohydrates, nucleic acids, proteins, lipids, cells and cell-derived products, metals and inorganic compounds, and metalorganic compounds, including naturally-derived and artificially-created substances.

In accordance with another embodiment, the present dissertation provides the use of separate, non-enzymatically digested and sieved ECM particles, including combinations of ECMs (e.g., SIS and UBM), which may also be added to the neutralized solution to homogenously incorporate ECM particles into the resulting bone graft material; the addition of these ECM particles may enhance the regenerative capacity of the resulting bone grafts as a result of providing native or near-native ECM protein structure and function. It is recognized that upon addition of any of the above-mentioned substances (e.g., pharmaceuticals, minerals, and/or ECM particles), neutral pH may need to be re-established, and the timing of gelation (paragraph below) may be affected. Finally, it is recognized that the properties of the final bone graft will change based on the composition of the material.

The homogenously dispersed mixture is poured into a mold, and the mold is tapped briefly to remove large air bubbles (Fig. 9). The mold containing the mixture is

then placed into any type of sealed chamber (e.g., pipette tip box; at least 10-times the volume of the mixture). The chamber is then placed into an incubator at 37 °C for about an hour. After about an hour, the mixture is solidified to the consistency of gelatin or thicker (Fig. 10), depending on the amount of inorganic components and/or other additives added. As a possible variant, porogens may be added to the mixture before incubation to alter the pore structure of the bone graft material (paragraph below).

The mold containing the gelatin like mixture of ECM and inorganic particles is transferred to a sealed container, which is then placed into a freezer at -20 °C for between 12 and 24 h. The frozen mixture still contained in the mold (-20 °C) is then lyophilized (vacuum approximately 0.1 mbar; collector temperature approximately -60 °C) for between 24 and 72 hours to dry the material and introduce pores. Examples of the final materials at this stage are shown in Fig, 11. As a possible variant, the freezing rate may be altered (e.g., near-instant freezing in liquid nitrogen) to alter the pore structure of the bone graft material.

It will be understood by those of ordinary skill in the art that the mold can have any shape and size for whatever grafting purpose is needed. The mold could have a long thin shape, or be round or oval or square or rectangular.

The lyophilized material created above is submerged in a dopamine solution (e.g., 2 mg dopamine HCl /mL of 10 mM tris buffer; adjusted to pH 8.5 with 0.1 N HCl or 0.1 N NaOH) at a concentration of 0.0075 g lyophilized material/mL dopamine solution for 5 h in the dark at room temperature (Fig. 12). To ensure uniform activation of the material with polydopamine, the container is flipped upside down once every 45 minutes. That is, the lyophilized material at a ratio of 1:2 (ECM: inorganic components) and greater (e.g.,

1:3, 1:4, 1:5, etc.) is denser than the dopamine solution and sinks; flipping the container upside down periodically ensures uniform activation of the material with polydopamine.

The polydopamine-activated material is then repeatedly washed with distilled water to remove heterogeneous clumps of dopamine at room temperature. The supernatant containing heterogeneous clumps of dopamine is removed from the container and replaced with distilled water. The polydopamine-activated material is allowed to stand in the water for 10 minutes; then, the container is flipped upside down, and the polydopamine-activated material is allowed to stand in the water for 10 more minutes.

The supernatant containing heterogenous clumps of material is removed, and fresh distilled water is then added to the container. The polydopamine-activated material is allowed to stand in the water for 10 minutes; then, the container is flipped upside down, and the polydopamine-activated material is allowed to stand in the water for 10 more minutes. The above step is repeated (approximately 2 additional times) until no heterogeneous clumps of material are present and the water remains colorless after the end of the rinsing (i.e., after 20 minutes of rinsing).

The polydopamine-activated material is transferred to a sealed container, which is then placed into a freezer at -20 ºC for between 12 and 24 h. The frozen material (-20 ºC) is then lyophilized (vacuum approximately 0.1 mbar; collector temperature approximately -60ºC) for between 24 and 72 hours to create the porous bone graft material. As a possible variant, the freezing rate may be altered (e.g., near-instant freezing in liquid nitrogen) to alter the pore structure of a bone graft material.

2.3.4 An Alternative Embodiment for Making the Bone Graft Materials (SIS as the ECM)

Lyophilized pepsin from porcine gastric mucosa (22.5 mg; 3,200-4,500 units/mg protein) was dissolved in 22.5 mL of 0.1 N HCl (1 mg pepsin/mL 0.1 N HCl) in a 50-Ml beaker equipped with a magnetic stir bar and sealed with Parafilm. The solution was stirred until the protease had completely dissolved and the solution was observed to be homogeneous (about 5 minutes). SIS (225 mg; 10 mg SIS/mL 0.1 N HCl), sieved to below 425 micrometers, was then added to the above solution. The beaker was then re-sealed with Parafilm and allowed to stir on a stir plate at room temperature for 24 h, at which point the ECM had completely dissolved and a near-colorless, homogenous viscous mixture without identifiable ECM particles was present. Dilute sodium hydroxide (2.22 mL; 0.1 N) was then added dropwise to neutralize the mixture while stirring. The solution was allowed to stir for 90 seconds at room temperature, after which the pH was confirm to be neutral. Next, 1800 mg of beta-tricalcium phosphate, sieved below 44 micrometers, (1:8 ratio of SIS:betatricalcium phosphate), was added to the neutralized mixture while stirring. The mixture was allowed to stir for 2 minutes, which resulted in a homogenous mixture of SIS and calcium phosphate particles, without distinction between the particles. It was observed that the mixture was beginning to gel/harden during this time.

The homogeneously dispersed mixture of SIS and beta-tricalcium phosphate was then poured into a 2” x 3” mold (Freshware SP-100RD). The mold was briefly tapped to remove air bubbles from the mixture, and then placed into a sealed Pipette tip box. This

box was then placed into an incubator at 37 oC for 1 h, after which time the white mixture had solidified to the consistency of thick gelatin. The mold containing the gelatin-like homogenous mixture of SIS and beta-tricalcium phosphate was then transferred to a sterile bag. The bag was sealed and then placed into a -20 oC freezer for 16 h. The frozen mixture contained in the mold (-20 oC) was then lyophilized (Labconco Freeze Dry System/Freezone 4.5; vacuum approximately 0.1 mbar; collector temperature approximately -60 oC) for 36 hours, yielding a beautiful white, porous, foam-like material containing homogenously dispersed SIS and beta-tricalcium phosphate.

The lyophilized material created above was then activated with polydopamine. First, the material was submerged in 270 mL of a dopamine solution (2 mg dopamine HCl/mL of 10 mM tris buffer; pH 8.5; 0.0075 g lyophilized material/mL dopamine solution) in a plastic container with lid for 3 h at room temperature. To ensure uniform activation of the material with polydopamine, the container (and bone graft material) was flipped upside down once every 45 minutes. It was noted that the lyophilized material sank in the prepared dopamine solution. The polydopamine-activated material was then repeatedly washed with distilled water to remove heterogeneous clumps of dopamine at room temperature. Namely, the supernatant dopamine solution was removed and replaced with distilled water. The polydopamine-activated material was allowed to stand in the water for 10 minutes; the container was then flipped upside down, and the polydopamine-activated material was allowed to stand in the water for 10 additional minutes. The supernatant was then replaced with fresh distilled water, and the rinsing process repeated for three additional times, after which point no heterogeneous clumps of material were observed and the water remained colorless. The polydopamine-activated material was

then transferred to a petri dish, which was then placed into a sterile plastic bag. These were placed into a -20 oC freezer for 16 h. The frozen material (-20 oC) was then lyophilized (Labconco Freeze Dry System/Freezone 4.5; vacuum approximately 0.1 mbar; collector temperature approximately -60 degrees Celsius) for 48 h, yielding a bone graft material.

Preliminary analyses support that 1) a 1:2 ratio of ECM to calcium phosphates promotes greatest bone growth (which corresponds to the most "biomimetic" composition, as native bone is comprised of ~1/3 organic components and ~2/3 inorganic components) and 2) polydopamine further promotes bone growth.

2.3.5 Materials with Growth Factors

In addition, as a possible variant, bone grafts of the present dissertation may be further activated with growth factors, which may have sustained and controlled release in the setting of polydopamine-activation. One or multiple different growth factors may be loaded onto the bone graft material as follows: the bone grafts are submerged in a solution (e.g., 10 mM tris buffer; pH 8.5) containing one or more growth factors (e.g., bone morphogenetic protein-2, vascular endothelial growth factor, and platelet-derived growth factor; concentration of 1 microgram growth factor/mL solution) in a container at room temperature for 5 h. The container is periodically flipped over to ensure homogenous loading of the growth factors. Unconjugated growth factors are then rinsed away by replacing the growth factor solution with a solution that is free of growth factors (e.g., 10 mM tris buffer; pH 8.5) and allowing the growth factor-loaded material to stand in the growth factor-free solution for 10 minutes before flipping the container and

allowing the material to stand for 10 additional minutes. The growth-factor loaded material is then transferred to a sealed container, which is then placed into a freezer at -20 ºC for between 12 and 24 h. The frozen material (-20 ºC) is then lyophilized (vacuum approximately 0.1 mbar; collector temperature approximately -60 ºC) for between 24 and 72 hours to create a porous, growth factor-loaded version of a bone graft material. Similar to that described above, the freezing rate may be altered (e.g., near-instant freezing in liquid nitrogen) to alter the pore structure of the bone graft. As yet another possible variant, bone grafts of the present work may be contacted with a solution containing growth factors, or other molecules, allowed to stand temporarily, and then implanted into a subject requiring a bone graft; this method may be identical to the preparation and use of the Infuse™ Bone Graft used clinically.

In accordance with yet another embodiment, the present dissertation involves mixing ECM particles (without enzymatic digestion) and inorganic particles with or without pharmaceuticals and/or minerals (described previously) in a minimal amount of dilute glycerol (e.g., 10% in water), which is a non-toxic, odorless, colorless and biodegradable viscous liquid that is classified by the FDA as a "Generally Recognized as Safe" multiple purpose food substance. Further, glycerol is used as a carrier of particulate powders in some commercially available bone grafts, including Grafton® DBM and Optium® DBM. This mixture may be used as a bone graft in this form – or, as a possible variant, transferred into a mold (e.g., 3" x 2"), which is then placed into a sealed container. The container is then placed into a freezer at -20 ºC for between 12 and 24 h. The frozen mixture is then lyophilized (vacuum approximately 0.1 mbar; collector temperature approximately -60 ºC) for between 24 and 72 hours to dry the material and

introduce pores. The lyophilized material is then activated with dopamine and re-lyophilized, as described above. All embodiments of the graft material may be coated with dopamine, and then re-lyophilized. In addition, all embodiments may include 3-D printing or electrospinning of the above-described bone graft components.

Scanning electron microscopy (e.g., microarchitecture) and biomechanical testing (e.g., elastic modulus) generally characterize bone graft materials of the present work.

Scanning electron microscopy (e.g., microarchitecture) and biomechanical testing (e.g., elastic modulus) generally characterize bone graft materials of the present work.

2.3.6 Biomimetic Bone Graft Substitute Materials with Small Molecules

In accordance with some embodiments, the bone graft materials incorporate various small molecules, like oxysterols, for example [21]. In a sealed beaker, ECM particles (e.g., porcine small intestinal submucosa, porcine urinary bladder matrix, etc.; and combinations thereof) are digested with pepsin from porcine gastric mucosa in dilute hydrochloric acid at room temperature for about 24 h while stirring, forming a digested ECM solution. The solution is neutralized with dilute sodium hydroxide and calcium phosphates are added, as described in previous embodiments. Then, an amount of oxysterol or combination of oxysterols is added to the solution. Various oxysterols may be added, as indicated in Table 2.2.

Table 2.2: Various types of oxysterols which can be used in the present grafts.

Oxysterol name	Change from cholesterol

20(*S*)-OHC	OH group at C_{20}(*S*)
22(*R*)-OHC	OH group at C_{22}(*R*)
22(*S*)-OHC	OH group at C_{22}(*S*)
Oxy4/Oxy34	OH group at C_{20}(*S*), single bond between C_5 and C_6, OH group at C_6(*S*)
Oxy18	OH group at C_{20}(*S*), single bond between C_5 and C_6, OH group at C_6(*S*), deuterated carbons at C_{22} and C_{23}
Oxy21/Oxy133	OH group at C_{20}(*S*), single bond between C_5 and C_6, OH group at C_6(*S*), n-hexane at C_{20}(*S*)
Oxy49	OH group at C_{20}(*S*), single bond between C_5 and C_6, OH group at C_6(*S)*, double bond between C_{25} and C_{27}

Without being held to any particular theory, it is believed that oxysterol-mediated osteoinduction occurs via activation of the Hedgehog (Hh) signaling pathway, which is known to be essential for normal bone development. In conical Hh signaling, the transmembrane protein Patched (Ptc) inhibits Smoothened (Smo) activity. However, upon binding Hh ligand, Ptc is inactivated, allowing for Smo activation. Activated Smo increases levels of the active forms of Gli transcription factors, which translocate to the nucleus and upregulate multiple genes, including those keys for bone formation. In a stereoisomer-specific manner, it is believed that oxysterols may directly activate Smo, thereby promoting Hh signaling and osteoinduction.

It is anticipated that the ratio of concentration of oxysterol to ECM can be about 10:1. In other words, the range of oxysterol amounts can vary from about 1% of the weight of

ECM to about 1000% of the weight of ECM. After about three minutes of stirring, a homogenous mixture comprising ECM, calcium phosphates, and oxysterols – and potentially any additional mineral and/or pharmaceutical agent – has been achieved, and the solution has begun to thicken. The mixture is poured into a mold, which is then placed into an incubator at 37 ºC. The thickened material is lyophilized, thereby forming a solid, porous, homogenously dispersed mixture comprising ECM, calcium phosphates, and oxysterols – and potentially any additional mineral and/or pharmaceutical agent – from a one-pot liquid ECM solution at room temperature and neutral pH (Figs. 18-19).

The material is then submerged in a solution of dopamine in tris buffer at pH 8.5, which infuses the material with polydopamine. This material is then lyophilized to create a bone graft of the present invention, namely, a solid, porous, homogenously dispersed mixture of ECM, calcium phosphates, and oxysterols – and potentially any additional mineral and/or pharmaceutical agent – that has been infused with polydopamine. This material may be subsequently submerged in a tris buffer solution at pH 8.5 containing growth factors (e.g., BMP-2, VEGF-165, PDGF-BB), loading growth factors onto the material. Lyophilizing this material creates a growth factor-loaded version of a bone graft of the present invention. In some embodiments, the dosage of BMP-2 is between about 1 μg to about 1000 μg, including, for example, dosages of 1, 2, 3, 4, 5, 6, 7, 8, 9 10 μg or more, such as 200, 300 400, 500, 600, 700, 800, 900, to 1000 μg.

2.3.7 Methods of Bone Grafting Using the Materials

A spinal fusion procedure is a surgical procedure used to create new bone about the spine to immobilize adjacent vertebrae. It is a surgical standard of care for patients with spinal instability, neoplasm, and/or spinal degenerative changes causing medication-refractory back and/or neck pain with or without spinal cord or nerve root compression. Successfully grafting bone about the spine is particularly challenging because, unlike in other applications of bone grafting (e.g., dental implants), the spine may be in motion during the grafting process. Small animal models of spinal fusion, including a posterolateral inter-transverse process fusion of the lumbar vertebrae L4 and L5, are clinically translatable models .

After Institutional Animal Care and Use Committee approval for the spinal fusion procedure has been received, a small animal (e.g., mouse, rat, or rabbit) is anesthetized using standard protocols. The hair on the back of the animal, in length being from approximately the thoracic vertebra T5 to the tail and in the width being that of the thoracic cavity, is then shaved using clippers. The skin is disinfected with 70% ethanol, and then sterilized with Betadine solution. A midline skin incision is then made beginning at approximately the thoracic vertebrae T9 and ending at the sacrum. Two paravertebral fascia incisions are then made, followed by careful dissection to expose the bony elements (e.g., transverse processes) of L4 and L5. Retractors and surgical dissecting instruments (e.g., forceps, scalpels, scissors, surgical curettes) may be used to facilitate the exposure (Fig. 15).

The bony elements are thoroughly cleaned of soft tissues which may interfere with bone grafting. In addition, the bony elements are decorticated (e.g., by cutting with a scalpel and/or using a drill) to expose red marrow; the red marrow may contain osteoprogenitor cells and osteoinductive factors (e.g., growth factors) to promote the spinal fusion. Further, decorticating the bony elements creates a vascular bed that may stimulate new vascular ingrowth in the implanted bone graft, facilitating the resorption and replacement of the bone graft with bone.

An embodiment of the bone graft material of the present invention is implanted over the decorticated bony elements and molded about the spine where new bone formation is desired. A bone graft which has enough flexibility to stay together while molding about the spine, without fragmenting, is desirable for spinal fusion (i.e., creating continuous, bridging bone between the adjacent vertebrae) (Fig. 16). The paraspinal muscles are then carefully returned to their normal, un-retracted position over the implanted bone graft, effectively sandwiching the bone graft between the decorticated bony elements and the paraspinal muscles. The fascia followed by skin is then closed using absorbable sutures, and the animal is then attended to for postoperative care while recovering from anesthesia (e.g., warming the animal and administering saline) before returning the animal to the cage.

The gold-standard method for evaluating spinal fusion clinically is via assessing for continuous, bridging bone formation between adjacent vertebrae via computed tomography – or, in animal models, micro-computed tomography. Continuous, bridging bone formation between the adjacent vertebrae is defined as a successful spinal fusion. A failed spinal fusion is defined as interrupted, or non-bridging, bone formation between

the adjacent vertebrae. The time-point of evaluation ranges across animal models; in the described spinal fusion procedure in a rat model, commonly evaluated time points are 8 and 10 weeks.

Bone grafting may be performed similarly (including preparing the site for bone grafting, implanting the bone graft, and closing the surgical site and providing post operative care) for a wide range of indications. These include the group consisting of a fracture; a knee problem requiring a knee replacement; a hip problem requiring a hip replacement; a missing tooth requiring bone for a dental implant; a bone issue requiring the treatment of critical-sized bony defects; a bone problem requiring a fusion procedure comprising a foot fusion, an ankle fusion, a finger fusion, a wrist fusion, and a spinal fusion; and a combination thereof.

2.3.8 Method of Spinal Fusion in a Preclinical Model

A posterolateral inter-transverse process spinal fusion of the lumbar vertebrae L4 and L5 in a rat model was performed after receiving Institutional Animal Care and Use Committee approval. A male Sprague Dawley rat at approximately 11 weeks of age was used. The rat was weighed to calculate the dosage of anesthetic (xylazine and ketamine, per standard protocol) required. The mass of the rat was 370 g, corresponding to 350-microliter of required anesthetic. The rat was injected with the anesthetic via intraperitoneal injection and then returned to its cage for the sedative to take effect. After approximately 5 minutes, the rat was observed to be fully asleep, as confirmed via lack of toe-pinch reflex. The hair on the back of the rat, in length being from approximately the thoracic vertebra T5 to the tail and in the width being that of the thoracic cavity, was then

shaved using clippers. The skin was then disinfected with 70% ethanol, and then sterilized with Betadine solution.

A midline skin incision was made beginning at approximately the thoracic vertebrae T9 and ending at the sacrum using an 11-blade. Two paravertebral fascia incision were then made using the same blade, followed by careful dissection to expose the bony elements (e.g., transverse processes) of the lumbar vertebrae L4 and L5. Retractors and surgical dissecting instruments, including forceps, scissors, and surgical curettes, were used to facilitate the exposure of the bony elements of L4 and L5. The transverse processes and lamina of L4 and L5 were then decorticated by cutting the bony elements with a 12-blade until punctate bleeding was observed. Afterwards, two pieces of bone graft material (i.e., SIS:beta-tricalcium phosphate in a 1:8 ratio), previously cut from a larger piece of graft (size per piece: 17 mm x 7 mm x 3 mm, L x W x H), were implanted over the decorticated bony elements, one per side, and molded about the spine where new bone formation was desired. Excellent flexibility and integrity of the grafts were noted on molding the material, especially when wetted with bone marrow from the decortication. The paraspinal muscles were then carefully returned to their normal, un-retracted position over the implanted bone grafts, effectively sandwiching the bone grafts between the decorticated bony elements and the paraspinal muscles. The fascia followed by skin was then closed using absorbable 3-0 sutures (Vicryl). The rat was administered 1 mL of normal saline subcutaneously and placed onto a heating pad until fully awake, after which point the animal was transferred to a clean cage with readily available access to food and water.

ECM can be isolated and used from any tissue/organ, and any such ECM is contemplated in the scope of the present invention. For example, our preliminary work suggests that SIS may be a superior source of ECM relative to UBM in the present invention. That is, versions of bone graft materials were created as described herein, with the only difference being the source of ECM – either from SIS or UBM. The SIS version demonstrated superior bone formation in a clinically translatable rat model of spinal fusion compared to the UBM version.

Chapter 3: Preclinical Evaluation of Materials

3.1 Comparison of BioMim-PDA to the Infuse™ Bone Graft

One embodiment of the materials, termed BioMim-PDA, was created following the methods described above. Briefly, ECM particles were generated from porcine small intestinal submucosa [22] (Figure 3.1).

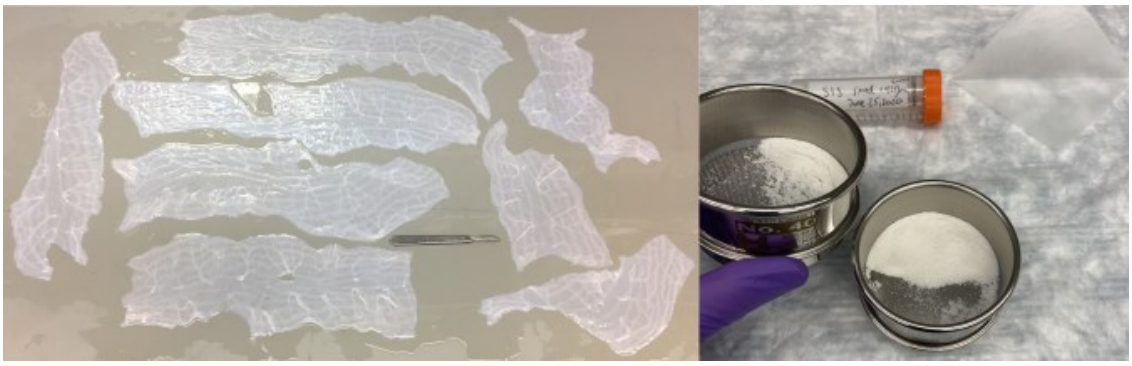

Figure 3.1: From fresh porcine small intestinal tissue, small intestinal submucosa (SIS) was mechanically separated (left), which was cryogenically milled and sieved to <425-μm to create a fine powder (right).

The ECM powder was then enzymatically digested using pepsin in a dilute HCl solution (pH 2; 10mg ECM/mL solution) for 24 hours (Figure 3.2) [23]. The pH was then adjusted to 7.4 to deactivate pepsin and initiate gelation. Immediately afterwards, an 80:20 biphasic mixture of beta-tricalcium phosphate and hydroxyapatite was added. This mixture was stirred for three minutes to form a homogenous mixture of ECM and calcium phosphates that was poured into a 2”×3” mold and warmed to 37°C (Figure 3.2).

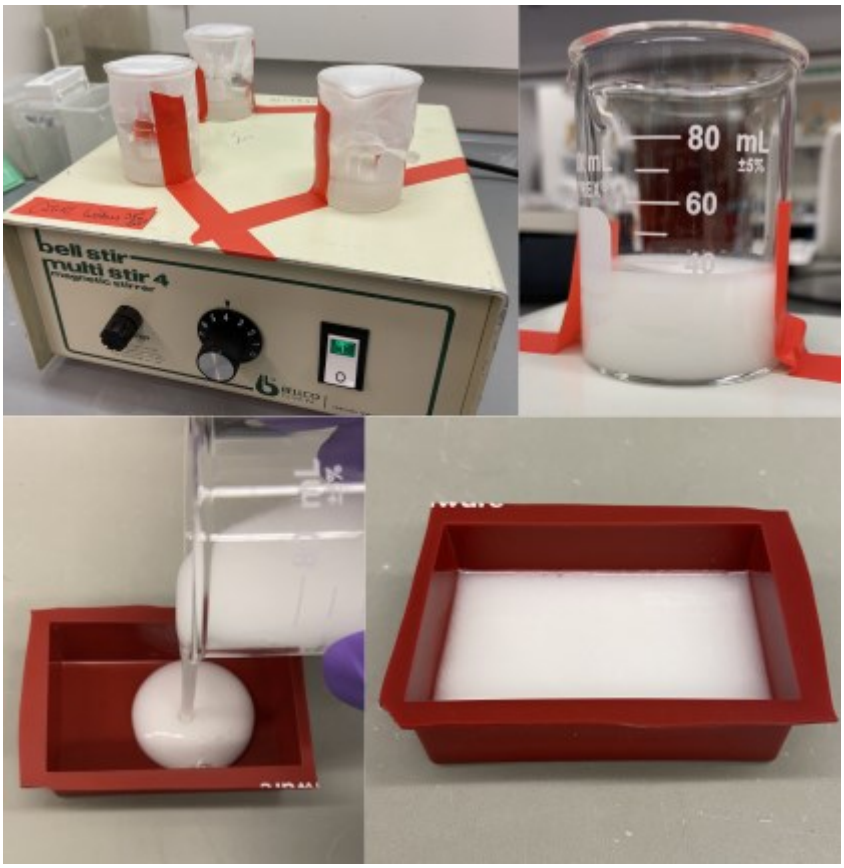

Figure 3.2: The SIS was enzymatically digested using pepsin in a dilute HCl solution for 24 hours on a magnetic stir plate (upper left), and then neutralized with dilute NaOH. Afterwards, an 80:20 biphasic mixture of beta-tricalcium phosphate and hydroxyapatite was homogenously incorporated with stirring (upper right), and the biomimetic mixture was then poured into a 2"×3" mold (lower left and lower right) and incubated at 37 ºC.

The mixture was then lyophilized, yielding a white, porous biomimetic bone graft material (Figure 3.3). The resultant scaffold was then divided into implant-sized pieces and infused with polydopamine by immersing them in a dopamine solution (2 mg dopamine HCl /mL of 10 mM tris buffer; pH 8.5) with gentle agitation for 5 hours. The polydopamine-infused grafts were washed with distilled water to remove

excess/unattached dopamine molecules and then lyophilized, yielding pieces of BioMim-PDA (Figure 3.3).

Figure 3.3: The biomimetic mixture was lyophilized, yielding a white, porous biomimetic bone graft material (upper left), which was then cut into pieces (upper right) for implantation in a rat model of spinal fusion. The pieces were instilled with polydopamine via immersion in a dopamine solution (lower left), washed with distilled water to remove excess dopamine molecules (lower middle), and then lyophilized to yield porous, homogenously dispersed solid mixtures of ECM and calcium phosphates instilled with polydopamine, called BioMim-PDA (lower right).

BioMim-PDA was compared directly against the collagen sponge in the Infuse™ Bone Graft in a rat model of posterolateral inter-transverse process spinal fusion of the lumbar vertebrae as described above. The purpose was to compare the spinal fusion ability of BioMim-PDA to that of the Infuse™ Bone Graft collagen sponge. Different amounts of rhBMP-2, which was obtained from the Infuse™ Bone Graft, were added in solution form to either BioMim-PDA or the collagen sponge 15 minutes before

implantation, which is analogous to the clinical use of the Infuse™ Bone Graft. Representative images of BioMim-PDA and the collagen sponge, before and after wetting with rhBMP-2, are shown in Figure 3.4.

Figure 3.4: Implant-sized pieces of BioMim-PDA (black) and collagen (white) are shown for comparison. The dry dimensions of all implants were 15mm×5mm (length×width). rhBMP-2 (0.2µg, 2.0µg, or 20µg) in aqueous solution (lower left) was uniformly distributed onto BioMim-PDA or collagen sponge, and the wetted graft was allowed to stand for 15 minutes before implantation; the wetted grafts assumed nearly identical physical dimensions (lower middle and lower right) and handleability.

Sixty rats were used in total, split equally between the graft types. Specifically, the following protocol was used, involving six different groups:

1. Collagen sponge from Infuse™ + 0.2µg rhBMP-2

2. BioMim-PDA + 0.2μg rhBMP-2
3. Collagen sponge from Infuse™ + 2.0μg rhBMP-2
4. BioMim-PDA + 2.0μg rhBMP-2
5. Collagen sponge from Infuse™ + 20μg rhBMP-2
6. BioMim-PDA + 20μg rhBMP-2

The bone graft substitute material was implanted over the decorticated transverse processes (Figure 3.5) and kept in place by reapproximation of the overlying paraspinal muscles.

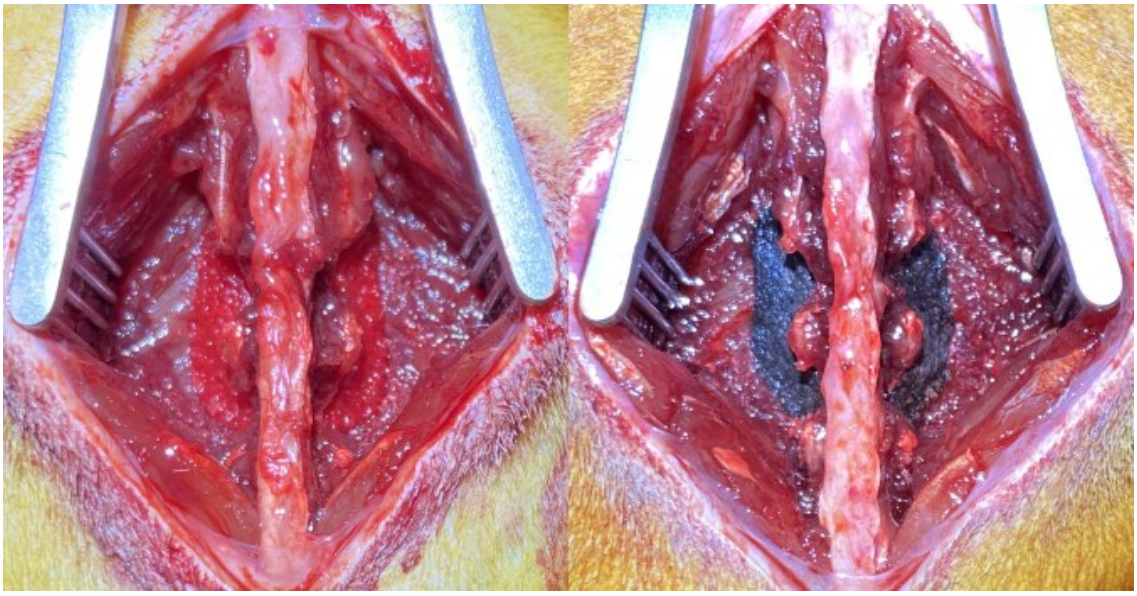

Figure 3.5: Grafts were evaluated using a clinically translatable rat model of spinal fusion. The L4-L5 transverse processes were exposed bilaterally and decorticated. rhBMP-2-adsorbed collagen sponge (left) or BioMim-PDA (right) was then implanted over the decorticated transverse processes bilaterally; both graft materials were easily moldable about the spine while retaining structural integrity.

3.1.1 Characterization of BioMim-PDA and a Collagen Sponge

Histology and scanning electron microscopy (SEM) were used to characterize the materials properties of BioMim-PDA and the collagen sponge from the Infuse® product. For histology, implant-sized pieces of BioMim-PDA and the collagen sponge were dehydrated in ethanol and then embedded in paraffin. Long-cut sections (7μm thick) were obtained, deparaffinized, and then rehydrated. Staining with hematoxylin and eosin (H&E), Masson's trichrome, and von Kossa stains was then performed. Separately, scanning electron microscopy (LEO field-emission, ZEISS; Oberkochen, Germany) was performed (100× magnification, accelerating voltage 1.0 kV) to investigate the micro-architectures of BioMim-PDA and the collagen sponge.

Comparative histological stains of BioMim-PDA and the collagen sponge using H&E, Masson's trichrome, and von Kossa stains are shown in Figure 3.6. As seen in the H&E and Masson's trichrome stains, both materials are porous and acellular, comprised of homogeneously distributed collagens. However, as seen in the von Kossa stains, BioMim-PDA, unlike the collagen sponge of the Infuse® Bone Graft, is also comprised of homogenously distributed calcium phosphates. Similarly, SEM imaging illustrates the porous, homogenous, and fibrillar architecture of both materials (Figure 3.7).

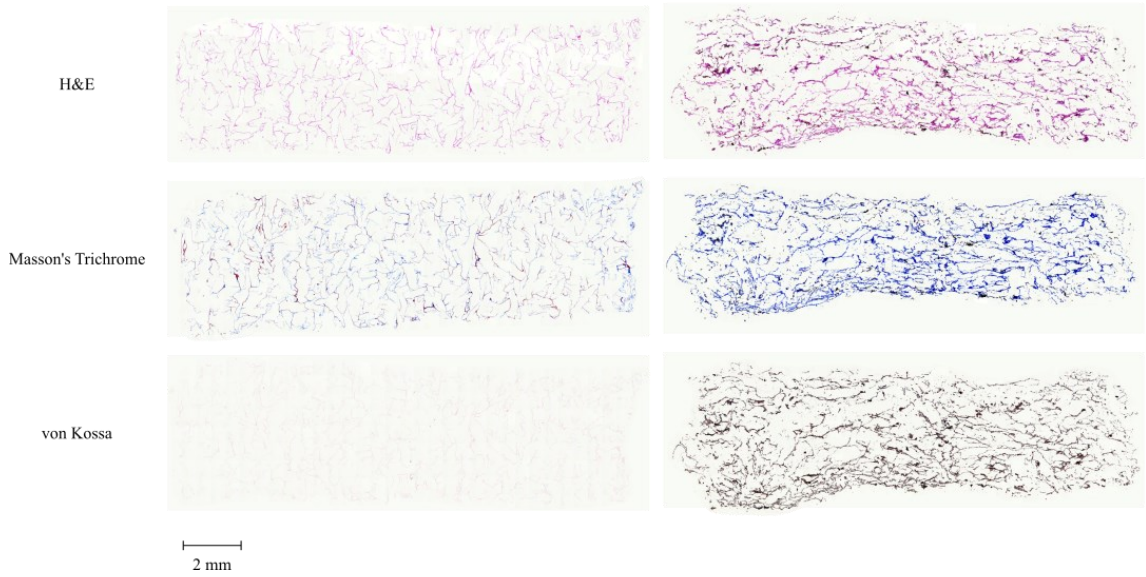

Figure 3.6: Comparative histological staining images of the collagen sponge (left) and BioMim-PDA (right) are shown. Both the collagen sponge and BioMim-PDA are porous, acellular, and comprised of homogeneously dispersed collagens (H&E and Masson's trichrome stains). However, in contrast to the collagen sponge, BioMim-PDA is also comprised of homogeneously dispersed calcium phosphates (von Kossa stains), which confer osteoconductivity and osteoinductivity.

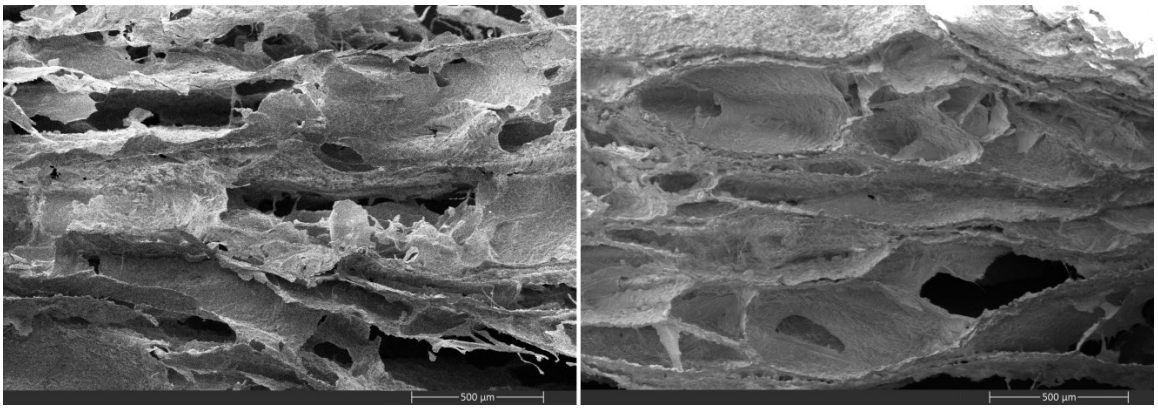

Figure 3.7: As shown via scanning electron microscopy, both the collagen sponge (left) and BioMim-PDA (right) are comprised of a porous, homogenous, fibrillar architecture. Magnification: 100× magnification; scale bar: 500µm.

3.1.2 Evaluation of Bone Formation

The spines were analyzed using high-resolution micro-computed tomography (μCT) and histology. Scans were performed using a SkyScan 1275 (Bruker; Billerica, MA) with 30μm slice thickness. Successful fusion was defined from the μCT imaging as continuous bridging bone from L4 to L5, bilaterally. In addition, quantitative μCT analysis of the fusion masses was performed using CTAn (Version 1.18.8.0; Bruker) to evaluate for bone volume (BV), as well as three parameters of bone quality: percent bone volume (bone volume divided by fusion mass volume; %BV), trabecular number (TN), and trabecular separation (TS). The fusion mass was defined as the volume of space lateral to the L4 and L5 vertebral bodies, including the transverse processes. Additionally, 3D reconstructions of the μCT scans were performed using CTVox (Version 3.3.0; Bruker) to macroscopically visualize the fusion masses.

After performing the μCT analysis, a representative sample from each group was prepared for histological analysis to evaluate the formation of bone, cartilage, and fibrous tissue. Samples were fixed in 4% paraformaldehyde solution, decalcified in RDO Rapid Decalcifier (Apex Engineering; Aurora, IL), dehydrated in ethanol, and then embedded in paraffin. Coronal sections were cut at a thickness of 7μm, deparaffinized, rehydrated, and then stained with H&E staining.

All sixty rats undergoing surgery survived until the end of the study (8 weeks postoperatively) and were evaluated. There were no wound site infections, instances of wound dehiscence, or other postoperative complications requiring intervention. The fusion rate was 100% in all groups, except for in Group 1 (collagen sponge + 0.2μg

rhBMP-2; 70%) and in Group 4 (BioMim-PDA + 2.0μg rhBMP-2; 90%). The one failed fusion in Group 4 was attributed to an intraoperative complication leading to excessive paraspinal bleeding and poor graft placement on one side, which was documented at the time of surgery. A unilateral fusion was observed on the unaffected side. There were no other instances of intraoperative complications. Significantly greater BV, %BV, and TN, as well as significantly smaller TS, were observed with the use of BioMim-PDA compared to use of the collagen sponge at each of the three concentrations of rhBMP-2. Furthermore, use of BioMim-PDA with 0.2μg rhBMP-2 led to significantly greater BV (125μm^3 vs. 101μm^3), %BV (32.6% vs. 23.9%), and TN (2.0/μm vs. 1.6/μm), as well as significantly smaller TS (0.39μm vs. 0.63μm), compared to the use of the collagen sponge with 2.0μg rhBMP-2. Similar observations were noted when comparing the use of BioMim-PDA with 2.0μg rhBMP-2 to use of the collagen sponge with 20μg rhBMP-2.

Representative μCT slices and corresponding histological images at the same level in the coronal plane are presented in Figure 3.8. Additionally, representative 3D reconstructions of the μCT data for each group are shown in Figure 3.9. Spinal fusions are shown for Groups 2-6, and a unilateral nonunion comprising fibrous tissue is shown for Group 1. As seen in both the radiographic and histological imaging, increases in the amount of rhBMP-2 led to decreases in %BV and TN, as well as an increase in TS, for both graft material types. The compositions, instructions for use, and efficacy of the materials are summarized in Table 3, below.

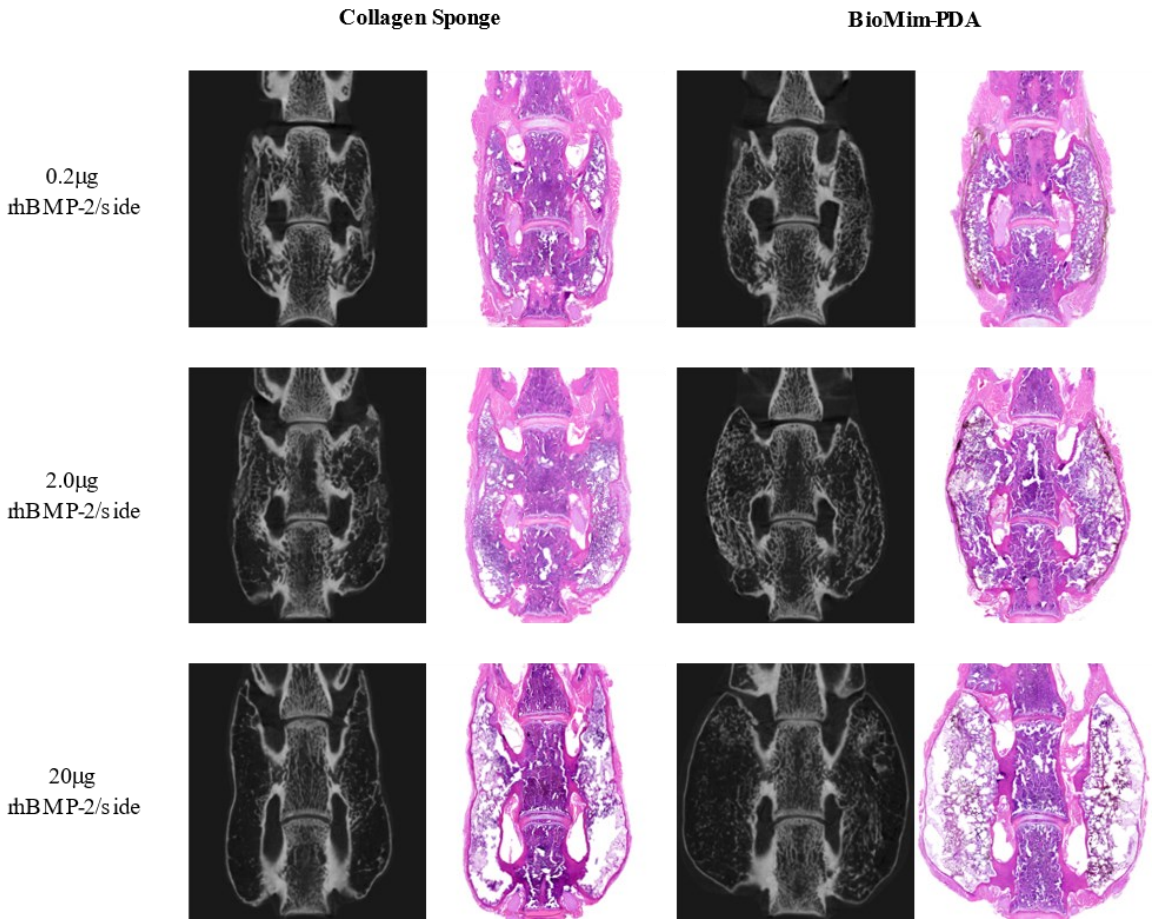

Figure 3.8: Representative µCT slices and corresponding H&E histological images at the same level in the coronal plane are shown. An example of a unilateral fibrous nonunion (right side) following use of the collagen sponge and 0.2µg rhBMP-2 is shown. Spinal fusions – defined as continuous bridging bone from L4 to L5, bilaterally – are shown for the remaining groups.

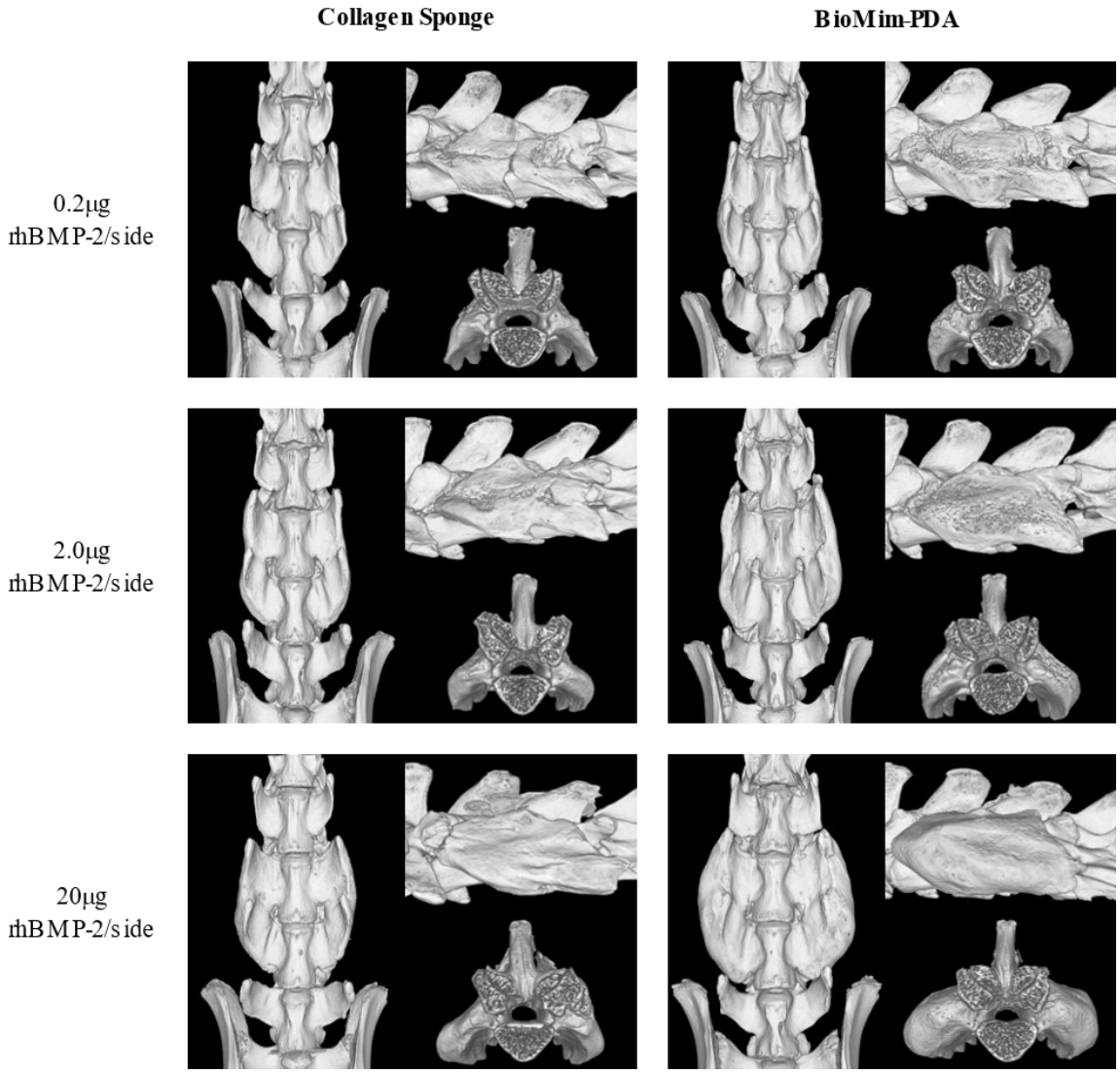

Figure 3.9: Representative 3D reconstructions of the µCT data are shown. Significantly greater bone volume and quality were observed with the use of BioMim-PDA compared to use of the collagen sponge at each of the three concentrations of rhBMP-2 evaluated. In addition, use of BioMim-PDA with 0.2µg rhBMP-2 led to significantly greater bone volume and quality compared to the use of the collagen sponge with 2.0µg rhBMP-2. Similar observations were noted when comparing the use of BioMim-PDA with 2.0µg rhBMP-2 to use of the collagen sponge with 20µg rhBMP-2.

Table 3.1: Summary of the compositions, instructions for use, and efficacy of the collagen sponge and BioMim-PDA when used with rhBMP-2 for bone grafting.

Bone Graft Substitute Material	**Scaffold Composition**	**Instructions for Use**	**Efficacy (evaluated in a rat model of spinal fusion)**
Collagen sponge + rhBMP-2 (Infuse® Bone Graft)	Bovine Type I collagen	Distribute rhBMP-2 onto the collagen sponge, wait 15 minutes, then implant	*Reference*
BioMim-PDA + rhBMP-2	Porcine small intestinal submucosa, calcium phosphates, and polydopamine	Same as above	Greater bone formation with 1/10 the dose of rhBMP-2 used with the collagen sponge

*Evaluated using a rat model of spinal fusion.

3.2 Evaluation of BioMim-Oxy-133

In two embodiments of the graft materials described herein, termed "BioMim-Oxy-133," the small molecule Oxy-133 was homogenously incorporated. Two versions of BioMim-Oxy-133 were created: "BioMim-Oxy-133-High," comprising Oxy-133 in an amount that was 3-times the amount of SIS, and "BioMim-Oxy-133-Low," comprising Oxy-133 in an amount that was 0.3-times the amount of SIS. Briefly, in two separate reactions, ECM particles were digested with pepsin from porcine gastric mucosa in dilute hydrochloric acid at room temperature for about 24 h while stirring, forming two digested ECM solutions. The solutions were neutralized with dilute sodium hydroxide and calcium phosphates (hydroxyapatite and beta-tricalcium phosphate) were added, as described in previous embodiments. Immediately thereafter, Oxy-133 (in the amounts described above) was added to the solutions, creating a "high-dose" version and "low-dose" version of BioMim-Oxy-133, as indicated above. After about three minutes of stirring,

homogenous mixtures comprising SIS, calcium phosphates, and Oxy-133 were created, and the solutions began to thicken. The mixtures were poured into separate molds, which were then placed into an incubator at 37 ºC. The thickened materials were lyophilized, yielding solid, porous, homogenously dispersed mixture comprising SIS, calcium phosphates, and Oxy-133 (Figure 3.10)

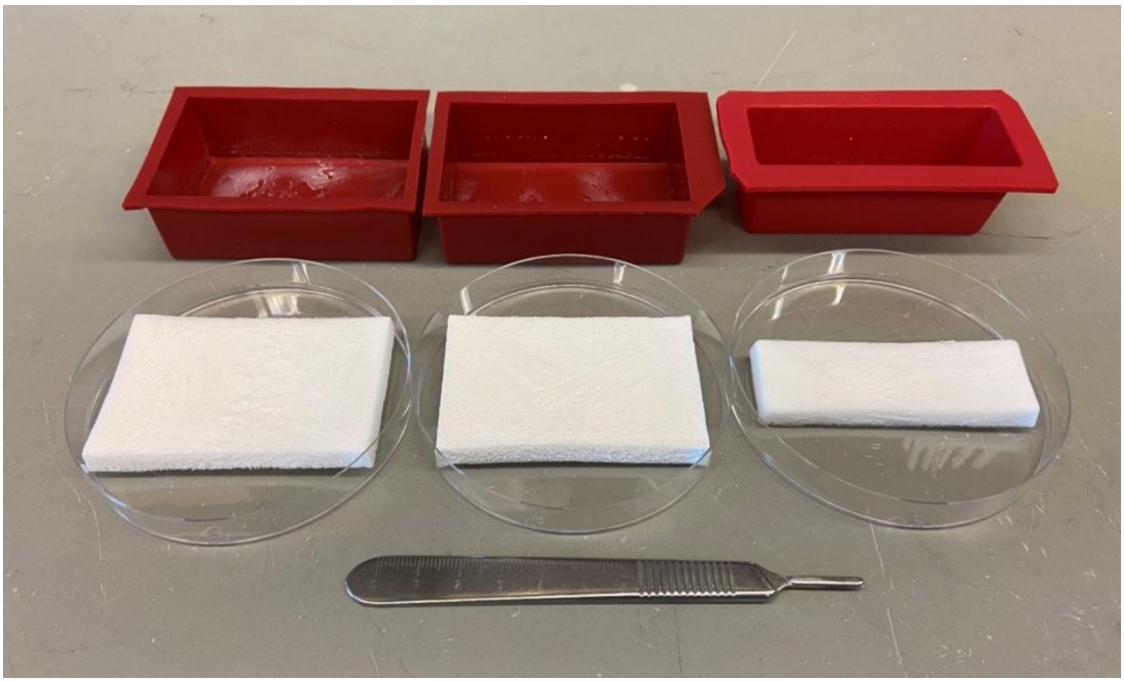

Figure 3.10: Comparative images of BioMim-Oxy-133-Low (left), BioMim-Oxy-133-High (middle), and biomimetic graft without Oxy-133 (right).

The high- and low-dose embodiments of BioMim-Oxy-133 were evaluated in a rat model of spinal fusion, as previously described (Figure 3.11). The fusion rates were 100% for both embodiments (8/8 animals for each embodiment), with greater bone formation observed with the high-dose embodiment (Figure 3.12). These results support the notion that the grafts described herein can be used as carrier scaffolds for a virtually

infinite array of bioactive additives. As the grafts are remodeled following implantation, they may release bioactive agents to orchestrate bone formation.

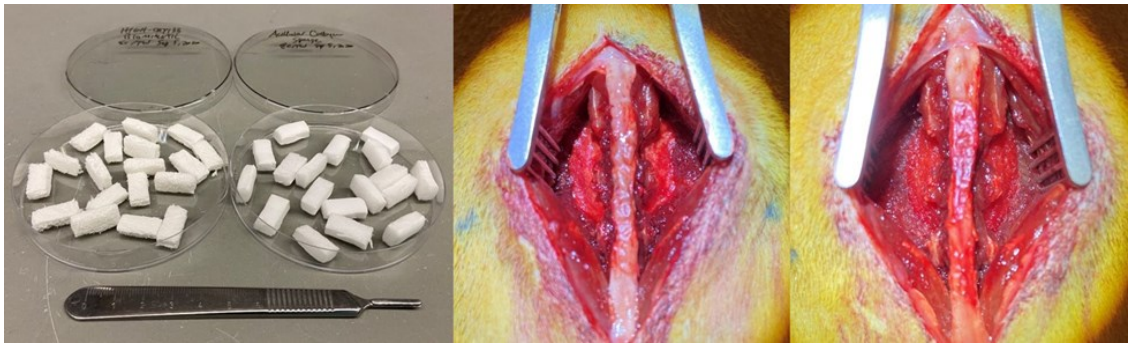

Figure 3.11: Comparative images of BioMim-Oxy-133-High (left) and collagen (right) as dry implant-sized pieces (left panel). Implanted in a rat model of spinal fusion, BioMim-Oxy-133-High (middle panel) and rhBMP-2-adsorbed collagen sponge (right panel) showed similar handleability and retention at the fusion site.

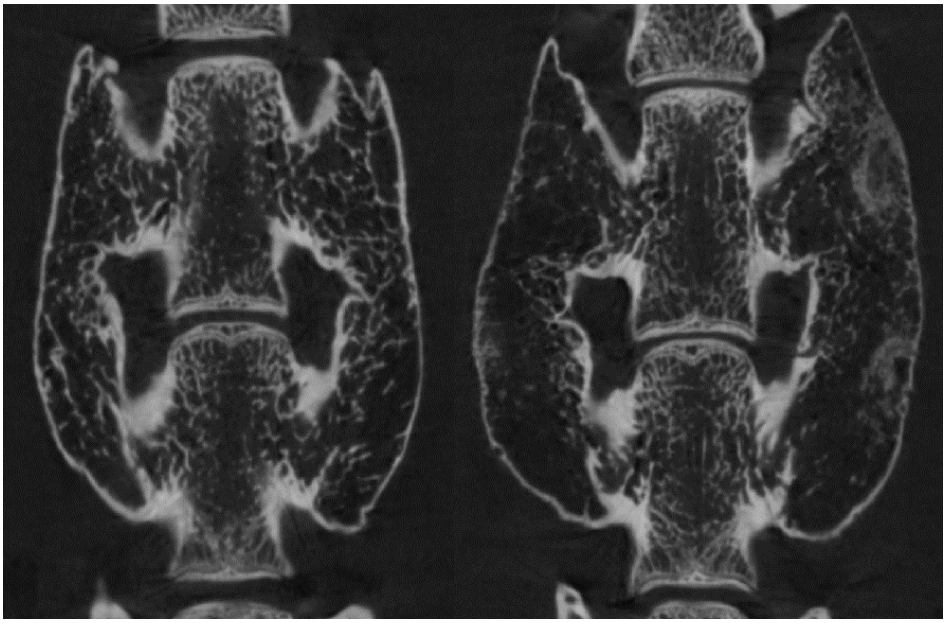

Figure 3.12: Representative μCT slices of the BioMim-Oxy-133-Low (left) and BioMim-Oxy-133-High (right) are shown. The fusion rates were 100% for both

embodiments (8/8 animals for each), with greater bone formation observed with the BioMim-Oxy-133-High.

Chapter 4: Discussion and Conclusions

Each year more than 500,000 Americans undergo spinal fusion for the treatment of axial neck/back pain, radiculopathy, and/or myelopathy [4]. The procedure involves instrumentation, like pedicle screws and rods, as well as bone graft to effect bony fusion. It is estimated that some 20-30% of these procedures fail to produce fusion, dependent upon patient comorbidities, surgical complexity, and bone graft material used [24, 25]. When the fusion fails, patients are often left with significant pain and limited treatment options, which typically consist of revision surgery [26].

The bone graft material is perhaps the most modifiable factor in spinal fusion procedures, and other bone grafting procedures, with different grafts producing different fusion outcomes [8, 9, 21, 27, 28]. Autologous bone, obtained either locally from the spine or taken from the iliac crest or rib, remains the gold standard graft material for fusion. However, autologous bone is naturally limited in supply, is associated with complications (e.g., pain) at the site of harvest, and does not guarantee fusion [27, 29]. Alternatives to autologous bone are called bone graft substitutes, which through advances in biomedical engineering offer extensive opportunities for the creation of novel and effective graft materials [8, 9, 21, 28, 30, 31].

A leading bone graft substitute material for spinal fusion is the Infuse™ Bone Graft (Medtronic; Memphis, TN), which over the past twenty years has generated billions of dollars in revenue in spine surgery. The product has not changed since its FDA approval for use in spinal fusion in 2002: it is comprised simply of a collagen sponge and an exogenously applied potent osteoinductive growth factor, recombinant human bone morphogenetic protein-2 (rhBMP-2). To use, the growth factor in aqueous solution is

dispensed onto the collagen sponge, which after allowed to stand for ≥15 minutes is implanted at the intended site of fusion.

Despite its commercial success, the Infuse™ Bone Graft is associated with significant harm, including osteolysis and cancer, and in general offers a fusion efficacy that is no better than that of autologous bone [12, 32]. The complications are believed to arise from the non-controlled, supraphysiologic burst release of rhBMP-2 from the collagen sponge following implantation [12, 33]. Furthermore, at approximately two weeks following the initial burst release of growth factor, there are essentially undetectable levels of rhBMP-2 remaining locally to continue promoting bone formation [34, 35]. Separately, the collagen sponge when used alone is known to produce minimal bone formation.

Through the present work, novel bone graft substitute materials were created to overcome the limitations of the available bone graft substitutes materials, including the Infuse™ Bone Graft. Specifically, we created a graft material with controlled, sustained delivery of rhBMP-2. Additionally, we replaced the collagen scaffold with a biomimetic construct comprising pro-regenerative extracellular matrix (ECM) and engineered calcium phosphates, which when used alone reliably produces new bone. In the process, we developed a novel method for creating a virtually infinite array of bone graft substitute materials based on a homogeneous solid mixture of ECM and calcium phosphates.

In one embodiment, ECM derived from porcine tissue is enzymatically digested in aqueous solution, subsequently pH-neutralized, and then combined with calcium phosphates, like hydroxyapatite and beta-tricalcium phosphate. The relative ratio of ECM to calcium phosphates dictate the material properties of the resultant graft material. The components are homogeneously mixed at room temperature during which time the mixture

thickens and is subsequently poured into a mold. This mixture in the approximate consistency of Jell-O is then incubated and subsequently lyophilized, yielding a solid, porous, homogeneous mixture of ECM and calcium phosphates.

Subsequently, the material is activated with dopamine, which confers controlled and sustained growth factor release. After re-lyophilization, a solid, porous homogeneous mixture of polydopamine-activated ECM and calcium phosphates is obtained, which is used in an identical fashion to the collagen sponge of the Infuse™ Bone Graft.

We employed a well-established and clinically translatable rat model of spinal fusion to evaluate fusion outcomes using this new bone graft substitute material [30, 31], iteratively altering the graft composition and re-revaluating to optimize bone formation. Once a satisfactory material producing bone on its own and having desirable handleability was identified, we directly compared this novel candidate material to the collagen sponge of the Infuse™ Bone Graft in the setting of different concentrations of rhBMP-2.

Briefly, a posterolateral inter-intertransverse process spinal fusion at the lumbar levels L4 and L5 was performed on 60 male Sprague Dawley rats weighing approximately 350 grams. Bilaterally about the spine, either the collagen sponge or the new material was implanted after wetted with an aqueous solution containing either 0.2 microgram, 2.0 micrograms, or 20 micrograms of rhBMP-2 and allowed to stand for 15 minutes, analogous to the clinical use of the Infuse™ Bone Graft.

The spines from all 60 rats were evaluated at the 8-week postoperative time point using quantitative micro-computed tomography (micro-CT). There was no interim mortality or wound site complication. Bone volume, bone volume per tissue volume, and trabecular structure was significantly enhanced using the novel bone graft substitute

material compared to the collagen sponge at each of the three concentrations of rhBMP-2 ($p < 0.01$ for each comparison). Spinal fusion was defined as bilateral bridging bone across the site of fusion. The overall fusion rate among animals treated with the novel graft material was 97% (29/30 spines); the failed fusion was a unilateral fusion, occurred in an animal treated with 2.0 micrograms of rhBMP-2, and was attributed to a documented intraoperative error. The overall fusion rate among animals treated with the collagen sponge was 90% (27/30 spines), with all three non-unions occurring in animals treated with 0.2 microgram of rhBMP-2.

The enhanced bone formation using the novel graft material compared to the collagen sponge may be due to a superior graft design. It is known that collagen when used alone is a poor and potentially inhibitory option for producing bone. The core advantage of collagen as a bone graft material is its handleability; it can be easily molded about the spine without breaking apart, effectively acting as a bridge for new bone. In contrast, calcium phosphates effectively produce new bone [27] but are brittle in scaffold form. In the present work, we created a novel material that combines the handleability of collagen and the osteoconductivity of calcium phosphates. Distinctly, instead of collagen we employ ECM, which has similar handleability to collagen and may uniquely orchestrate a pro-regenerative healing response.

The use of polydopamine confers additional advantages. It has been suggested that the complications associated with the Infuse™ Bone Graft arise secondary to the non-controlled burst release of rhBMP-2, which may exceed more than one million-times the normal physiologic concentrations.[12, 33, 35] Furthermore, spinal fusion is a process that requires months to complete under normal circumstances. In the Infuse™ Bone Graft,

virtually all rhBMP-2 is leached from the collagen sponge in as little as two weeks.[34, 35] It naturally follows that the local, sustained release of rhBMP-2 may enhance bone formation. Polydopamine essentially acts as a biological adhesive for growth factors, providing controlled early and sustained release.[36-38] Interestingly, our initial work demonstrated enhanced bone formation using polydopamine-activated biomimetic grafts compared to their non-activated counterparts, even in the absence of exogenously applied growth factors. This may be due to the ability of polydopamine to concentrate innately produced growth factors at the site of fusion, as well as promote requisite vascular formation through its degradation products.[39]

A key advantage of our biomimetic bone graft technology is the ability to create a virtually infinite array of embodiments, as the grafts are created from an aqueous solution at room temperature and physiologic pH. At their core, the grafts comprise a homogeneous mixture of ECMs and calcium phosphates – but essentially any other small molecule and/or mineral may be homogenously incorporated into the graft during the synthetic process. In fact, the synthetic process is similar to baking a cake, in which essentially any ingredient may be added to the cake batter and homogeneously incorporated by mixing, before baking. The "baking" of our grafts occurs at physiologic temperature, preserving the bioactivity of entrapped small molecules. As the graft degrades following implantation, the bioactive small molecules are released to affect biological processes.

To illustrate this key advantage, we created two additional biomimetic bone grafts that incorporated an investigational osteoinductive small molecule, Oxy-133. The difference between the two grafts was the amount of small molecule incorporated. These materials were created in an identical manner to those described above – except that the

small molecule was added to the “cake batter” during the synthetic process. The resultant materials were solid, porous, homogeneous mixtures of ECM, calcium phosphates, and Oxy-133.

We evaluated these novel materials in the same rat model of spinal fusion. Impressively, even without polydopamine or exogenous rhBMP-2, these materials yielded a spinal fusion rate of 100% (16/16 spines) at the 8-week postoperative time point. Furthermore, the version comprising the greater amount of small molecule produced significantly more bone relative to its lower-dose counterpart. These exciting results suggest that our biomimetic bone grafts can effectively function as drug-delivery vehicles for bone grafting. These results also compare favorably to prior work using the rat model of spinal fusion, in which a recent meta-analysis of 26 studies determined an overall fusion rate of only 42.5% using autologous iliac crest bone graft.

There are several limitations to the present work, including evaluating the efficacy of the graft materials in preclinical models only, which may not reflect clinical reality. In all animal investigations, we followed “Animal Research: Reporting of In Vivo Experiments” guidelines [40], as well as utilized an established preclinical model of spinal fusion, to strengthen the quality of the preclinical investigations and the conclusions that can be drawn from them. In addition, we did not evaluate the release kinetics of rhBMP-2 from the collagen sponge or BioMim-PDA, which may be useful for optimizing physiologic drug delivery parameters. Despite these limitations, we believe that our biomimetic bone graft technology, described in this thesis work, has the potential to change the standard of care for patients requiring a bone graft. We developed a platform technology for creating a virtually infinite array of biomimetic bone grafts

comprising homogeneously dispersed ECMs and calcium phosphates – as well as potentially any additional small molecule and/or mineral. We are actively optimizing existing formulations for bone grafting, as well as exploring novel embodiments, including biomimetic bone grafts that incorporate antibiotics. Ultimately, we hope that our work may be used to help patients in need of a bone graft.

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