

little as 7 days, the mice receiving the cell support composition of the present invention show improvement over control animals not receiving the compositions of the present invention.

What is Claimed is:

1. A cell support composition comprising in parts by weight 5-50% laminin, wherein laminin type 1 and laminin type 3 are not present in any amount, 5-50% collagen, 1-10% nidogen, 5-60% heparan sulfate proteoglycan, and 1-10% entactin.
2. The cell support composition of claim 1, wherein laminin type 5 is not present in any amount.
3. The cell support composition of claim 1, wherein collagen type IV is present.
4. The cell support composition of claim 1, wherein collagen type I is present.
5. The cell support composition of claim 1, wherein collagen type VI is present.
6. The cell support composition of claim 3, wherein collagen type VI is present.
7. The cell support composition of claim 4, wherein collagen type VI is present.
8. The cell support composition of claim 7, wherein collagen type VI is present.
9. The cell support composition of claim 1, wherein the heparan sulfate proteoglycan comprises perlecan, wherein the perlecan is present in the cell support composition in an amount from about 5% to about 60% by weight.
10. The cell support composition of claim 1, further comprising titan protein.
11. The cell support composition of claim 1, further comprising myosin 7.
12. The cell support composition of claim 1, further comprising actinin protein.
13. The cell support composition of claim 1, wherein the cell support composition promotes the maintenance of plasticity of stem cells in cell culture.
14. The cell support composition of claim 1, wherein the cell support composition polymerizes at a temperature of at least about 37°C within about 1 hour.
15. The cell support composition of claim 1, wherein the cell support composition is polymerized.

16. The cell support composition of claim 15, wherein the polymerized cell support composition is in form of nanofibers.
17. A cell culture surface comprising the cell support composition of claim 15.
18. A method of maintaining the plasticity of stem cells, comprising culturing the stem cells on a cell culture surface comprising the cell support composition of claim 1, wherein the cell support composition has been polymerized prior to culturing the stem cells thereon .
19. The method of claim 18, wherein the stem cells are adult stem cells (ASCs).
20. The method of claim 18, wherein the stem cells are embryonic stem cells (ESCs).
21. The method of claim 18, wherein the stem cells are induced pluripotent cells (iPCs).
22. The method of claim 18, wherein the cell support composition is in the form of nanofibers.
23. A method of treating tissue comprising administering to a subject in need of treatment the cell support composition of claim 1.

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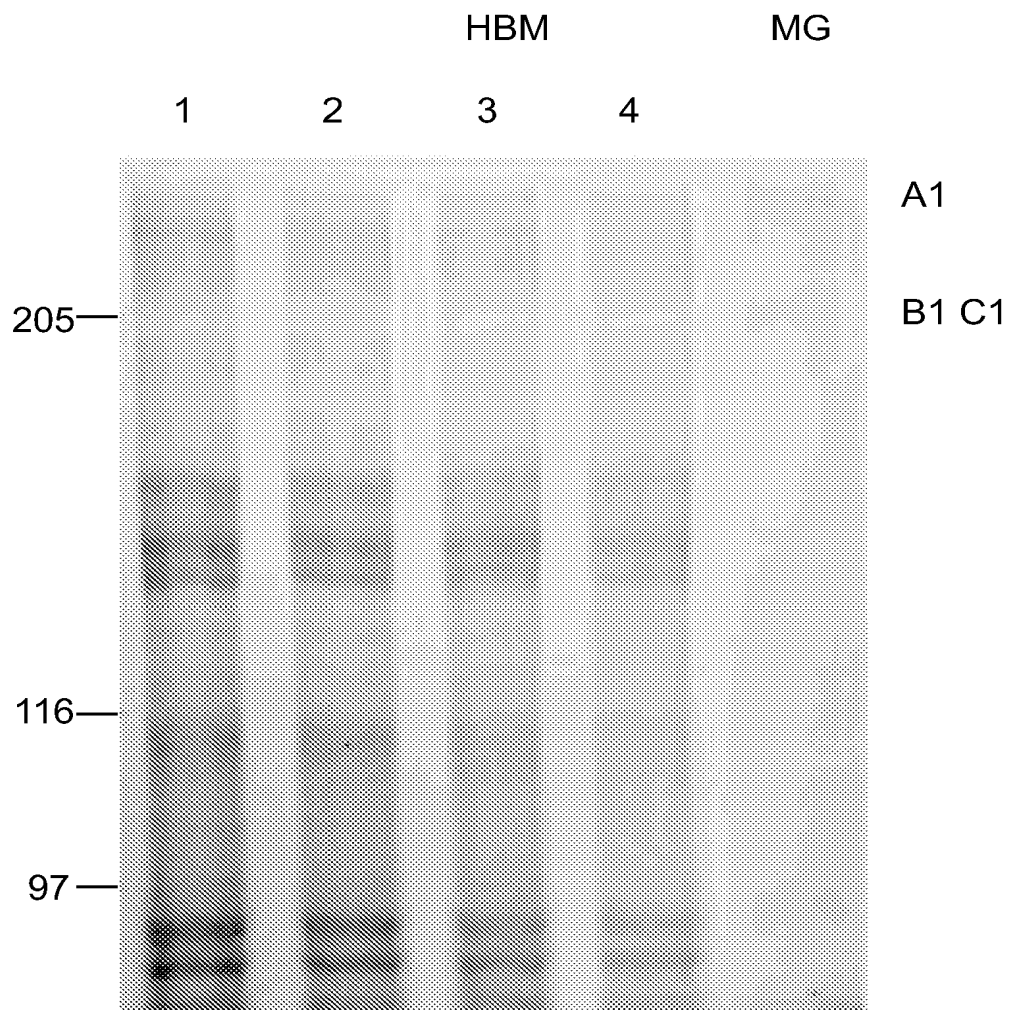
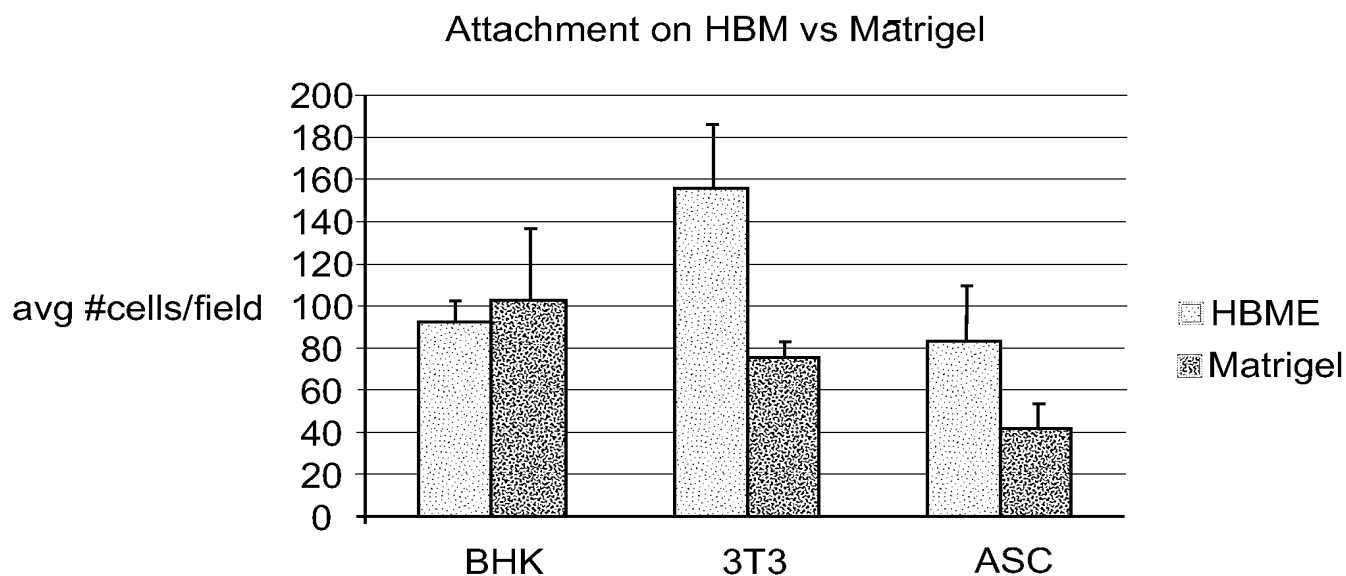


FIG. 1

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CELL NAME	CELL TYPE	SPECIES	HBM/MG ATTACHMENT
3T3	FIBROBLAST-LIKE	MOUSE	2.08
BHK	KIDNEY EPITHELIAL	HAMSTER	0.90
ASC	MULTIPOTENT SC	HUMAN	1.76
hIPSc	PLURIPOTENT SC	HUMAN	2.30

FIG. 2

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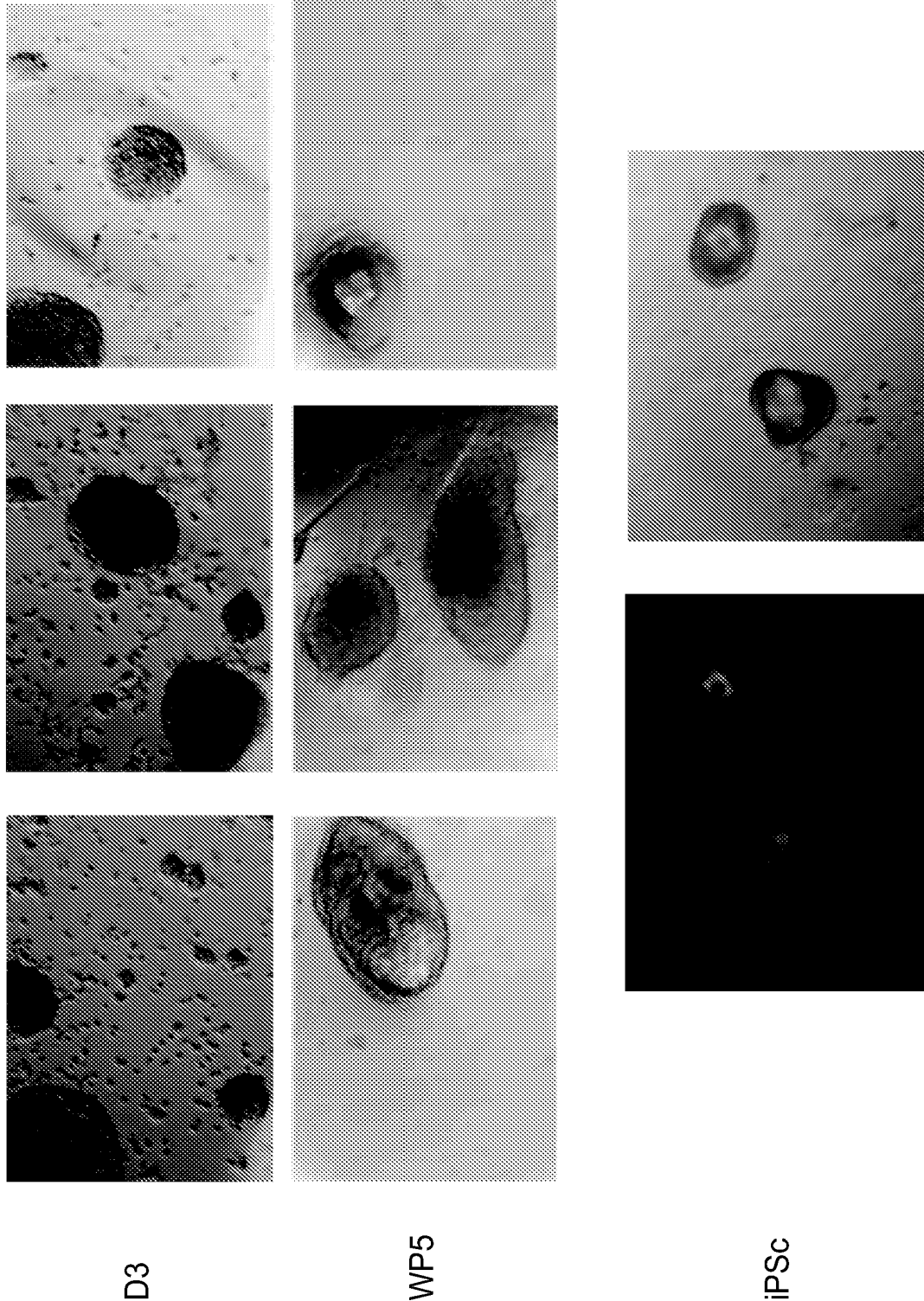


FIG. 3

GelatinMatrigelHBM

D3

WP5

iPSc



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(54) Title: BMP PEPTIDES & METHODS OF USE

(57) Abstract: The invention relates to truncated growth factors and variants thereof. The invention also relates to methods of making and using the truncated growth factors. The invention further relates to compositions including a protease and a growth factor comprising a bone morphogenic protein (BMP) or a variant thereof. The invention also relates to methods of using the composition.

BMP PEPTIDES & METHODS OF USE**Cross-Reference to Related Applications**

[0001] This application claims priority to U.S. Provisional Application Nos. 61/530,706, 61/530,715, 61/530,727, and 61/536,035, filed September 2, 2011, September 2, 2011, September 2, 2011, and September 18, 2011, respectively, all of which are incorporated herein by reference in their entireties.

Background of the Invention

[0002] The invention relates to truncated growth factors and variants thereof. The invention also relates to methods of making and using the truncated growth factors.

[0003] The invention relates to compositions including a protease and a growth factor comprising a bone morphogenic protein (BMP) or a variant thereof. The invention also relates to methods of using the composition.

Summary of the Invention

[0004] The invention relates to isolated peptides of 137 residues or less, with the peptide comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.

[0005] The invention relates to isolated peptides of 119 residues or less, with the peptide comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:34.

[0006] The invention relates to isolated peptides of 119 residues or less, with the peptide comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:35.

[0007] The invention also relates to fusion proteins comprising the inventive peptides described herein fused to a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO:40, 41, 42, 43, 44, 45, 46, 47, 48, 54, or 55.

[0008] The invention also relates to methods of promoting osteoinductive, chondroinductive, ligament differentiating, and/or tendon differentiating activity, with the methods comprising contacting cells with the inventive peptides described herein.

[0009] The invention also relates to methods of administering the inventive peptides described herein to matrices.

[0010] The invention also relates to methods of increasing osteogenesis, chondrogenesis, or ligament/tendon genesis in cells comprising administering to the cells the inventive peptides described herein.

[0011] The invention also relates to methods of increasing a cellular growth factor activity comprising administering to the cells at least one protease and the growth factor comprising SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27, 28, 34 or 35. In some embodiments, the composition comprises two or more proteases.

[0012] The invention relates to compositions comprising at least one protease and a growth factor, wherein the growth factor is (i) a bone morphogenic protein (BMP) or (ii) an isolated peptide comprising an amino acid sequence at least 92% identical to any of amino acid sequences of SEQ ID NO: 16-35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity. In some embodiments, the composition comprises two or more proteases.

[0013] The invention also relates to methods of increasing a growth factor activity comprising administering to the cells the composition described herein.

Brief Description of the Drawings

[0014] FIGURE 1 depicts myoblast expression of alkaline phosphatase after being cultured with rhBMP-2 modified with trypsin. Myoblasts treated with rhBMP-2 modified with trypsin showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-2.

[0015] FIGURE 2 depicts myoblast expression of alkaline phosphatase after being cultured with rhBMP-2 modified with collagenase. Myoblasts treated with rhBMP-2 modified with collagenase showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-2. When fractionated via HPLC, fractions A42-44, A45-46 and A47-49 induced the highest levels of alkaline phosphatase in myoblasts.

[0016] FIGURE 3 depicts myoblast expression of alkaline phosphatase after being cultured with rhBMP-2 control, purified modified rhBMP-2 and non-purified modified rhBMP-2.

[0017] FIGURE 4 depicts myoblast expression of alkaline phosphatase after being cultured with rhBMP-2 control and purified modified rhBMP-2 in the presence of no additional protease, dispase, inactive dispase, collagenase, or inactive collagenase.

[0018] FIGURE 5 depicts myoblast expression of alkaline phosphatase after being cultured with modified rhBMP-4. At a concentration of 30 ng/mL, cells treated with modified rhBMP4 expressed significantly higher AP activities than those treated with unmodified mature rhBMP-4.

[0019] FIGURE 6 depicts myoblast expression of alkaline phosphatase after being cultured with different concentrations of unmodified or dispase-modified rhBMP-5. Myoblasts treated with dispase modified rhBMP-5 showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-5.

[0020] FIGURE 7 depicts myoblast expression of alkaline phosphatase after being cultured with different concentrations of unmodified or collagenase-modified rhBMP-5. Myoblasts treated with collagenase modified rhBMP-5 showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-5.

[0021] FIGURE 8 depicts the expression of alkaline phosphatase in myoblast after being cultured with purified modified rhBMP-5 alone, or the same concentration of purified modified rhBMP-5 with the addition of dispase or collagenase.

[0022] FIGURE 9 depicts myoblast expression of alkaline phosphatase after being cultured with modified rhBMP-7. Myoblasts treated with modified rhBMP-7 showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-7. When fractionated via HPLC, fractions 43 induced the highest levels of alkaline phosphatase in myoblasts.

[0023] FIGURE 10 depicts expression of alkaline phosphatase in myoblasts after being cultured with various concentrations of modified rhBMP-7.

[0024] FIGURE 11 depicts expression of alkaline phosphatase in myoblasts after being cultured with unmodified rhBMP-7 or trypsin-modified rhBMP-7.

[0025] FIGURE 12 depicts in vitro alkaline phosphatase expressions by myoblast cells cultured with non-treated rhBMP-7 control, dispase modified rhBMP-7, or dispase modified rhBMP-7 with the addition of collagenase in culture media.

[0026] FIGURE 13 depicts the expression of alkaline phosphatase in C2C12 cells after being cultured with various concentrations of unmodified or dispase modified rhBMP-13 (rhGDF-6). The alkaline phosphatase activity induced by dispase-modified mature rhBMP-13 was about 300 - 350 times higher than that induced by unmodified mature rhBMP-13 control.

[0027] FIGURE 14 depicts the expression of alkaline phosphatase in C2C12 cells after being cultured with various concentrations of unmodified or collagenase modified mature rhBMP-13 (rhGDF-6). The alkaline phosphatase activity induced by collagenase-modified mature rhBMP-13 was about 0.7 - 20 times higher than that induced by unmodified mature rhBMP-13 control.

[0028] FIGURE 15 depicts myoblast expression of alkaline phosphatase after being cultured with various concentrations of unmodified or modified rhBMP-14 (rhGDF-5). Myoblasts treated with dispase modified rhBMP-14 showed significantly greater alkaline phosphatase activity over myoblasts treated with unmodified rhBMP-14 control. The level of alkaline phosphatase activity increase was dose-dependent upon rhBMP-14 concentration.

[0029] FIGURE 16 depicts the expression of alkaline phosphatase in C2C12 cells after being cultured with purified modified rhBMP-14 alone, or the same concentration of purified modified rhBMP-14 with the addition of dispase. The alkaline phosphatase activity in the cells induced by the purified modified rhBMP-14 in combination with dispase was about 235% higher than that induced by the purified modified rhBMP-14 alone.

[0030] FIGURE 17 depicts the expression of alkaline phosphatase in C2C12 cells after being cultured with non-purified modified rhBMP-14, or the same concentration of non-purified modified rhBMP-14 with addition of dispase or collagenase. The alkaline phosphatase activity in the cells induced by the non-purified modified rhBMP-14 in combination with dispase or collagenase were about 120% and 45% higher than that induced by the non-purified modified rhBMP-14 alone, respectively.

Detailed Description of the Invention

[0031] The invention relates to compositions comprising at least one protease and a growth factor comprising a bone morphogenic protein (BMP) or a variant thereof. In some embodiments, the growth factors are bone morphogenic proteins ("BMP," "hBMP," or "rhBMP"). The term "bone morphogenetic protein" refers to a protein belonging to the BMP family of the TGF- β superfamily of proteins (BMP family) based on DNA and amino acid sequence homology. A protein belongs to the BMP family according to this invention when it has at least 50% amino acid sequence identity with at least one known BMP family member within the conserved C-terminal cysteine-rich domain which characterizes the BMP protein family. Members of the BMP family may have less than 50% DNA or amino acid sequence identity overall. Examples of BMPs include, but are not limited to, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and BMP-16, *e.g.*, amino acid sequences of SEQ ID NO:1-15, respectively. In further embodiments, the growth factors are isolated peptides comprising mature peptide sequences of the BMPs. In additional embodiments, the growth factors are isolated peptides comprising modified growth factors described below. In yet additional embodiments, the growth factors are fusion proteins comprising the modified growth factors described herein fused to a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequences of SEQ ID NO: 36-55.

[0032] In some embodiments, the BMPs used in the present invention expressly exclude BMP-2, BMP-4, BMP-5, BMP-7, BMP-13, and BMP-14. In some embodiments, the BMPs used in the present invention expressly exclude BMP-2, BMP-4 and BMP-7.

[0033] In one specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP is not BMP-2, BMP-4, BMP-5, BMP-7, BMP-13, or BMP-14. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP is not BMP-2, BMP-4, BMP-5, BMP-7, BMP-13, or BMP-14. In the embodiments where the BMP is not BMP-2, BMP-4, BMP-5, BMP-7, BMP-13, or BMP-14, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0034] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP is not BMP-2, BMP-4 or BMP-7. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP is not BMP-2, BMP-4 or BMP-7. In the embodiments where the BMP is not BMP-2, BMP-4 or BMP-7, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0035] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, residue 317-454 of SEQ ID NO: 4, residue 293-431 of SEQ ID NO: 6, and residue 336-455 of SEQ ID NO: 12, and residue 382-501 of SEQ ID NO: 13. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, residue 317-454 of SEQ ID NO: 4, residue 293-431 of SEQ ID NO: 6, residue 336-455 of SEQ ID NO: 12, and residue 382-501 of SEQ

ID NO: 13. In the embodiments where the BMP does not have any amino acid sequence of 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, residue 317-454 of SEQ ID NO: 4, residue 293-431 of SEQ ID NO: 6, residue 336-455 of SEQ ID NO: 12, and residue 382-501 of SEQ ID NO: 13, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0036] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, and residue 293-431 of SEQ ID NO: 6. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, and residue 293-431 of SEQ ID NO: 6. In the embodiments where the BMP does not have any amino acid sequence of residue 283-396 of SEQ ID NO: 1, residue 293-408 of SEQ ID NO: 3, and residue 293-431 of SEQ ID NO: 6, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0037] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3, 4, 6, 12, and 13. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3, 4, 6, 12, and 13. In the embodiments where the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3, 4, 6, 12, and 13, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0038] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3 and 6. In further

embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3 and 6. In the embodiments where the BMP does not have any amino acid sequence of SEQ ID NO: 1, 3 and 6, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0039] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 16-28 and 30-35. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 16-28 and 30-35. In the embodiments where the BMP does not have any amino acid sequence of SEQ ID NO: 16-28 and 30-35, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0040] In another specific embodiment, the composition of the present invention comprises at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 16-19 and 30-33. In further embodiments, the method of the present invention comprise treating a cell with the composition comprising at least one protease and a growth factor, wherein the growth factor is a bone morphogenic protein (BMP), wherein the BMP does not have any amino acid sequence of SEQ ID NO: 16-19 and 30-33. In the embodiments where the BMP does not have any amino acid sequence of SEQ ID NO: 16-19 and 30-33, the composition optionally comprises two or more proteases, the protease is optionally selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof, the protease is optionally collagenase or dispase.

[0041] The terms “peptide,” “polypeptide” and “protein” are used interchangeably herein. As used herein, an “isolated polypeptide” is intended to mean a polypeptide that has been completely or partially removed from its native environment. For example, polypeptides that have been removed or purified from cells are considered isolated. In addition, recombinantly produced polypeptides

molecules contained in host cells are considered isolated for the purposes of the present invention. Moreover, a peptide that is found in a cell, tissue or matrix in which it is not normally expressed or found is also considered as “isolated” for the purposes of the present invention. Similarly, polypeptides that have been synthesized are considered to be isolated polypeptides. “Purified,” on the other hand is well understood in the art and generally means that the peptides are substantially free of cellular material, cellular components, chemical precursors or other chemicals beyond, perhaps, buffer or solvent. “Substantially free” is not intended to mean that other components beyond the novel peptides are undetectable. The peptides of the present invention may be isolated or purified.

[0042] The amino acid sequences of SEQ ID NO:1-15 represent the full length “prepropeptide” of some exemplary BMPs, BMP-2-16, respectively. In some embodiments, the BMP is formed as a full length prepropeptide, which usually contains, from N-terminus to C-terminus, a signal sequence, a propeptide domain and the “mature” peptide. Table 1 shows exemplary BMP sequences and the “mature” peptide sequences in the sequences.

[0043] Table 1. Mature peptide sequences in various types of BMPs.

Type of BMP	Genbank Accession No. or UniProt Accession No.	Mature Peptide
BMP-2	NP_001191	residue 283-396 of SEQ ID NO: 1
BMP-3	P12645	residue 363-472 of SEQ ID NO: 2
BMP-4	P12644	residue 293-408 of SEQ ID NO: 3
BMP-5	NP_066551	residue 317-454 of SEQ ID NO: 4
BMP-6	P22004	residue 375-513 of SEQ ID NO: 5
BMP-7	P18075	residue 293-431 of SEQ ID NO: 6
BMP-8	P34820	residue 243-402 of SEQ ID NO: 7
BMP-9	Q9UK05	residue 319-429 of SEQ ID NO: 8
BMP-10	O95393	residue 316-424 of SEQ ID NO: 9
BMP-11	O95390	residue 298-407 of SEQ ID NO: 10
BMP-12	P43029	residue 316-461 of SEQ ID NO: 11
BMP-13	NP_001001557	residue 336-455 of SEQ ID NO: 12
BMP-14	NP_000548	residue 382-501 of SEQ ID NO: 13
BMP-15	O95972	residue 267-392 of SEQ ID NO: 14
BMP-16	AAO31471	residue 171-280 of SEQ ID NO: 15

[0044] Normally, the BMP family of growth factors is translated into the full length prepropeptide. Through subsequent post-translational processing, the signal peptide and the propeptide domains are cleaved with the remaining portion of the peptide recognized as the “mature” form of the growth factor. After processing, the “mature” form of the protein normally dimerizes and active homodimeric or even heterodimeric bone morphogenetic proteins are secreted from cells and this dimer, in general, binds to its heteromeric receptor complex composed of BMP receptor type I and

BMP receptor type II, to initiate the cell signaling cascade typically associated with the specific BMP activity.

[0045] As used herein, “modified growth factor,” “modified BMP,” “truncated growth factor,” or “truncated BMP” refers to truncations of any form of a wild type BMP disclosed herein, *i.e.* BMP 2-16, including the full length prepropeptide, the propeptide (the full length peptide without the signaling sequence) and the mature form of the disclosed BMP. The invention therefore provides the composition comprising a protease and an isolated peptide, with the peptide comprising an amino acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to any of the amino acid sequences of these modified growth factors. In one specific embodiment, the isolated peptide comprises an amino acid sequence at least 92% identical to any of the amino acid sequences of the modified growth factors. In further embodiments, the peptide according to some embodiments of the present invention comprise an amino acid sequence 100% identical to any of the amino acid sequences of the modified growth factors. In still even more embodiments, the peptides consist of any of the amino acid sequences of the modified growth factors.

[0046] The modified growth factors in accordance with some embodiments of the present invention include, but are not limited to, truncations of the mature BMPs. Specifically, Table 2 below shows exemplary truncations of various mature BMPs.

[0047] Table 2. Exemplary truncations of various mature BMPs

Type of BMP	Truncated Sequence
BMP-2	SEQ ID NO: 16-18
BMP-4	SEQ ID NO: 19
BMP-5	SEQ ID NO: 20-28
BMP-6	SEQ ID NO: 29
BMP-7	SEQ ID NO: 30-33
BMP-13	SEQ ID NO: 34
BMP-14	SEQ ID NO: 35

[0048] In one embodiment, the isolated peptide is 113, 112, 111, 110, 109, 108, 107, 106, 105, 104 or 103 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequences of SEQ ID NO:16, 17, or 18.

[0049] In another embodiment, the isolated peptide is 115, 114, 113, 112, 111, 110, 109, 108, 107 or 106 residues or less with each peptide independently comprising an amino acid sequence at least

75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:19.

[0050] In another embodiment, the isolated peptide is 137, 136, 135, 134, 133, 132, 131, 130, 129, 128, 127, 126, 125, 124, 123, 122, 121, 120, 119, 118, 117, 116, 115, 114, 113, 112, 111, 110, 109 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.

[0051] In another embodiment, the isolated peptide is 138, 137, 136, 135, 134, 133, 132, 131, 130, 129, 128, 127, 126, 125, 124, 123, 122, 121, 120, 119, 118, or 117 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:29.

[0052] In another embodiment, the isolated peptide is 138, 137, 136, 135, 134, 133, 132, 131, 130, 129, 128, 127, 126, 125, 124, 123, 122, 121, 120, 119, 118, 117, 116, 115, 114, 113 or 112 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:30, 31, 32 or 33.

[0053] In another embodiment, the isolated peptide is 119, 118, 117, 116, 115, 114, 113, 112, 111, 110, 109 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:34.

[0054] In another embodiment, the isolated peptide is 119, 118, 117, 116, 115, 114, 113, 112, 111, 110, 109, 108, 107, 106 residues or less with each peptide independently comprising an amino acid sequence at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:35.

[0055] In some embodiments, the peptide variants described herein still retain their ability to specifically interact, at least partially, with its corresponding bone morphogenic protein receptor (BMPR). In additional embodiments, the peptide variants described herein are functional and capable of promoting osteoinductivity, stimulating proliferation of osteoblasts and/or promoting osteogenesis. In further embodiments, the peptide variants described herein are functional and capable of promoting chondroinductivity, stimulating proliferation of chondrocytes and/or promoting chondrogenesis. In even more embodiments, the modified growth factors of the present invention have enhanced activity

compared to the unmodified growth factors. In further embodiments, the compositions comprising a protease and the growth factor described herein have enhanced activity compared to compositions comprising the growth factor without the protease.

[0056] A polypeptide having an amino acid sequence at least, for example, about 95% “identical” to a reference amino acid sequence, *e.g.*, SEQ ID NO:16-35, is understood to mean that the amino acid sequence of the polypeptide is identical to the reference sequence except that the amino acid sequence may include up to about five modifications per each 100 amino acids of the reference amino acid sequence. In other words, to obtain a peptide having an amino acid sequence at least about 95% identical to a reference amino acid sequence, up to about 5% of the amino acid residues of the reference sequence may be deleted or substituted with another amino acid or a number of amino acids up to about 5% of the total amino acids in the reference sequence may be inserted into the reference sequence. These modifications of the reference sequence may occur at the N- terminus or C-terminus positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among amino acids in the reference sequence or in one or more contiguous groups within the reference sequence.

[0057] As used herein, “identity” is a measure of the identity of nucleotide sequences or amino acid sequences compared to a reference nucleotide or amino acid sequence. In general, the sequences are aligned so that the highest order match is obtained. “Identity” per se has an art-recognized meaning and can be calculated using well known techniques. While there are several methods to measure identity between two polynucleotide or polypeptide sequences, the term “identity” is well known to skilled artisans (Carillo (1988) J. Applied Math. 48, 1073). Examples of computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux (1984) Nucleic Acids Research 12, 387), BLASTP, ExPASy, BLASTN, FASTA (Atschul (1990) J. Mol. Biol. 215, 403) and FASTDB. Examples of methods to determine identity and similarity are discussed in Michaels (2011) Current Protocols in Protein Science, Vol. 1, John Wiley & Sons.

[0058] In one embodiment of the present invention, the algorithm used to determine identity between two or more polypeptides is BLASTP. In another embodiment of the present invention, the algorithm used to determine identity between two or more polypeptides is FASTDB, which is based upon the algorithm of Brutlag (1990) Comp. App. Biosci. 6, 237-245). In a FASTDB sequence alignment, the query and reference sequences are amino sequences. The result of sequence alignment is in percent identity. In one embodiment, parameters that may be used in a FASTDB alignment of amino acid sequences to calculate percent identity include, but are not limited to: Matrix=PAM, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1,

Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject amino sequence, whichever is shorter.

[0059] If the reference sequence is shorter or longer than the query sequence because of N-terminus or C-terminus additions or deletions, but not because of internal additions or deletions, a manual correction can be made, because the FASTDB program does not account for N-terminus and C-terminus truncations or additions of the reference sequence when calculating percent identity. For query sequences truncated at the N- or C-termini, relative to the reference sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminus to the reference sequence that are not matched/aligned, as a percent of the total bases of the query sequence. The results of the FASTDB sequence alignment determine matching/alignment. The alignment percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score can be used for the purposes of determining how alignments “correspond” to each other, as well as percentage identity. Residues of the reference sequence that extend past the N- or C-termini of the query sequence may be considered for the purposes of manually adjusting the percent identity score. That is, residues that are not matched/aligned with the N- or C-termini of the comparison sequence may be counted when manually adjusting the percent identity score or alignment numbering.

[0060] For example, a 90 amino acid residue query sequence is aligned with a 100 residue reference sequence to determine percent identity. The deletion occurs at the N-terminus of the query sequence and therefore, the FASTDB alignment does not show a match/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the reference sequence (number of residues at the N- and C-termini not matched/total number of residues in the reference sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched (100% alignment) the final percent identity would be 90% (100% alignment – 10% unmatched overhang). In another example, a 90 residue query sequence is compared with a 100 reference sequence, except that the deletions are internal deletions. In this case the percent identity calculated by FASTDB is not manually corrected, since there are no residues at the N- or C-termini of the subject sequence that are not matched/aligned with the query. In still another example, a 110 amino acid query sequence is aligned with a 100 residue reference sequence to determine percent identity. The addition in the query occurs at the N-terminus of the query sequence and therefore, the FASTDB alignment may not show a match/alignment of the first 10 residues at the N-terminus. If the remaining 100 amino acid residues of the query sequence have 95% identity to the entire length of the reference sequence, the N-terminal addition of the query would be ignored and the percent identity of the query to the reference sequence would be 95%.

[0061] The invention further embraces other species, preferably mammalian, homologs with amino acid sequences that correspond to the modified growth factors of the present invention. Species homologs, sometimes referred to as “orthologs,” in general, share at least 35%, 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity with the human version of the growth factors. Such corresponding sequences account for the modified growth factor from across a variety of species, such as canine, feline, mouse, rat, rabbit, monkey, etc. of BMP. In another embodiment, the invention provides novel peptides whose sequences correspond to the sequence of SEQ ID NO:16-35 and retain at least some minimal function.

[0062] Modified growth factor products with an additional methionine residue at position -1 (Met⁻¹-peptide) are contemplated, as are variants with additional methionine and lysine residues at positions -2 and -1 (Met⁻²-Lys⁻¹-peptide). Variants of the modified growth factor with additional Met, Met-Lys, or Lys residues (or one or more basic residues in general) are particularly useful for enhanced recombinant protein production in bacterial host cells.

[0063] Variants resulting from insertion of the polynucleotide encoding the modified growth factor into an expression vector system are also contemplated. For example, variants (usually insertions) may arise from when the amino terminus and/or the carboxy terminus of modified growth factor is/are fused to another polypeptide.

[0064] In another aspect, the invention provides deletion variants wherein one or more amino acid residues in the modified growth factor peptide are removed. Deletions can be effected at one or both termini of the modified growth factor peptide, or with removal of one or more non-terminal amino acid residues of the modified growth factor peptide. Deletion variants, therefore, include all fragments of the modified growth factor peptide.

[0065] Within the confines of the disclosed percent identity, the invention also relates to substitution variants of disclosed polypeptides of the invention. Substitution variants include those polypeptides wherein one or more amino acid residues of truncated growth factor are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature; however, the invention embraces substitutions that are also non-conservative. Conservative substitutions for this purpose may be defined as set out in the tables below. Amino acids can be classified according to physical properties and contribution to secondary and tertiary protein structure. A conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are set out in below.

[0066] Table 3: Conservative Substitutions

Side Chain Characteristic	Amino Acid
<u>Aliphatic</u>	
Non-polar	Gly, Ala, Pro, Iso, Leu, Val
Polar-uncharged	Cys, Ser, Thr, Met, Asn, Gln
Polar-charged	Asp, Glu, Lys, Arg
<u>Aromatic</u>	His, Phe, Trp, Tyr
<u>Other</u>	Asn, Gln, Asp, Glu

[0067] Alternatively, conservative amino acids can be grouped as described in Lehninger (1975) Biochemistry, Second Edition; Worth Publishers, pp. 71-77, as set forth below.

[0068] Table 4: Conservative Substitutions

Side Chain Characteristic	Amino Acid
<u>Non-polar (hydrophobic)</u>	
Aliphatic:	Ala, Leu, Iso, Val, Pro
Aromatic:	Phe, Trp
Sulfur-containing:	Met
Borderline:	Gly
<u>Uncharged-polar</u>	
Hydroxyl:	Ser, Thr, Tyr
Amides:	Asn, Gln
Sulfhydryl:	Cys
Borderline:	Gly
<u>Positively Charged (Basic):</u>	Lys, Arg, His
<u>Negatively Charged (Acidic)</u>	Asp, Glu

[0069] And still other alternative, exemplary conservative substitutions are set out below.

[0070] Table 5: Conservative Substitutions

Original Residue	Exemplary Substitution
Ala (A)	Val, Leu, Ile
Arg (R)	Lys, Gln, Asn
Asn (N)	Gln, His, Lys, Arg
Asp (D)	Glu
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
His (H)	Asn, Gln, Lys, Arg
Ile (I)	Leu, Val, Met, Ala, Phe
Leu (L)	Ile, Val, Met, Ala, Phe
Lys (K)	Arg, Gln, Asn
Met (M)	Leu, Phe, Ile
Phe (F)	Leu, Val, Ile, Ala
Pro (P)	Gly
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser
Val (V)	Ile, Leu, Met, Phe, Ala

[0071] The polypeptides according to the present invention may or may not be involved in a dimer. In one embodiment, the invention provides dimers, wherein the dimers comprise at least one of the novel, modified growth factors described herein. In one specific embodiment, the dimers are homodimers of the novel modified growth factors. In another embodiment, the dimers are heterodimers comprising at least one of the novel modified growth factors. As used herein, a “heterodimer” means a dimer of two peptides, wherein the amino acid sequences of the peptides are

not 100% identical to each other. Thus, a heterodimer may include a modified growth factor peptide of the present invention dimerized with a normal, "mature" version of the same growth factor.

[0072] As used herein in the specification and in the claims, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. In some embodiments, at least one refers, for example, to 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

[0073] It should be understood that the definition of peptides or polypeptides of the invention is intended to include polypeptides bearing modifications other than insertion, deletion, or substitution of amino acid residues. By way of example, the modifications may be covalent in nature, and include for example, chemical bonding with polymers, lipids, other organic and inorganic moieties. Such derivatives may be prepared to increase circulating half-life of a polypeptide, or may be designed to improve the targeting capacity of the polypeptide for desired cells, tissues or organs. Similarly, the invention further embraces modified growth factor peptides that have been covalently modified to include one or more water-soluble polymer attachments such as polyethylene glycol, polyoxyethylene glycol or polypropylene glycol.

[0074] Modified growth factor compositions in which the modified growth factor is linked to a polymer are included within the scope of the present invention. The polymer may be water soluble to prevent precipitation of the protein in an aqueous environment, such as a physiological environment. Suitable water-soluble polymers may be selected from the group consisting of, for example, polyethylene glycol (PEG), monomethoxypolyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, polypropylene glycol homopolymers, a polypropylene oxide/ethylene oxide copolymer polyoxyethylated polyols (*e.g.*, glycerol) and polyvinyl alcohol. The selected polymer is usually modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, so that the degree of polymerization may be controlled. Polymers may be of any molecular weight, and may be branched or unbranched, and mixtures of such polymers may also be used. When the chemically modified NgR polymer is destined for therapeutic use, pharmaceutically acceptable polymers will be selected for use.

[0075] Pegylation of modified growth factor peptides may be carried out by any of the pegylation reactions known in the art. For example, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer). One water-soluble polymer for pegylation of polypeptides is polyethylene glycol (PEG), including, but not limited to bi-functional PEGs. As used herein, "polyethylene glycol" is

meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (Cl-CIO) alkoxy- or aryloxy-polyethylene glycol.

[0076] Chemical derivatization of modified growth factor may be performed under any suitable conditions used to react with a biologically active substance with an activated polymer molecule. Methods for preparing pegylated modified growth factor will generally comprise the steps of (a) reacting the polypeptide with polyethylene glycol, such as a reactive ester or aldehyde derivative of PEG, under conditions whereby modified growth factor polypeptide becomes attached to one or more PEG groups, and (b) obtaining the reaction products. It will be apparent to one of ordinary skill in the art to select the optimal reaction conditions or the acylation reactions based on known parameters and the desired result.

[0077] Pegylated and other polymer modified growth factor polypeptides may generally be used to treat conditions that may be alleviated or modulated by administration of the modified growth factor polypeptides described herein. However, the chemically-derivatized polymer: modified growth factor polypeptide molecules disclosed herein may have additional activities, enhanced or reduced biological activity, or other characteristics, such as increased or decreased half-life, as compared to the nonderivatized molecules. The modified growth factor polypeptides, fragments thereof, variants and derivatives, may be employed alone, together, or in combination with other pharmaceutical compositions. For example, cytokines, growth factors, antibiotics, anti-inflammatories and/or chemotherapeutic agents may be co-administered as is appropriate for the indication being treated.

[0078] The present invention provides compositions comprising purified polypeptides of the invention. Preferred compositions comprise, in addition to the polypeptide of the invention, a pharmaceutically acceptable (*i.e.*, sterile and non-toxic) liquid, semisolid, or solid diluent that serves as a pharmaceutical vehicle, excipient or medium. Any diluent known in the art may be used. Exemplary diluents include, but are not limited to, water, saline solutions, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, glycerol, calcium phosphate, mineral oil and cocoa butter.

[0079] In one embodiment, the growth factors according to the present invention are fusion proteins that comprise at least a first and a second fusion peptides. The fusion partners are, generally speaking, covalently bonded to one another via a typical amine bond between the fusion peptides, thus creating one contiguous amino acid chain. In some embodiments, the first peptide of the fusion protein comprises the modified growth factor described herein. In further embodiments, the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:36-55.

[0080] In one embodiment, the first fusion peptide comprises a modified growth factor from BMP-2, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:36, 37 or 38. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:36, 37 or 38.

[0081] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-4, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:39. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:39.

[0082] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-5, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:40, 41, 42, 43, 44, 45, 46, 47 or 48. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:40, 41, 42, 43, 44, 45, 46, 47 or 48.

[0083] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-6, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:49. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:49.

[0084] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-7, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:50, 51, 52 or 53. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:50, 51, 52 or 53.

[0085] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-13, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:54. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:54.

[0086] In another embodiment, the first fusion peptide comprises a modified growth factor from BMP-14, and the second fusion peptide comprises an amino acid sequence that does not include the amino acid sequence of SEQ ID NO:55. In more specific embodiments, the second fusion of the present invention comprises an amino acid sequence that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% identical to SEQ ID NO:55.

[0087] Other types of fusion proteins provided by the present invention include but are not limited to, fusions with secretion signals and other heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the protein to improve stability and persistence in the host cell, during purification or during subsequent handling and storage.

[0088] Additional fusion proteins include fusions for enhancing translocation of the protein across cell membranes. For example, Tat is an 86-amino acid protein involved in the replication of human immunodeficiency virus type 1 (HIV-1). The HIV-1 Tat transactivation protein is efficiently taken up by cells, and it has been demonstrated that low concentrations (nM) are sufficient to transactivate a reporter gene expressed from the HIV-1 promoter. Exogenous Tat protein is able to translocate through the plasma membrane and reach the nucleus to transactivate the viral genome. Tat peptide-mediated cellular uptake and nuclear translocation have been demonstrated in several systems. Chemically coupling a Tat-derived peptide (residues 37-72 of Tat) to several proteins results in their internalization in several cell lines or tissues (Fawell (1994) Proc. Natl. Acad. Sci. USA 91, 664-668).

[0089] It is well-known that a region of the Tat protein centered on a cluster of basic amino acids is responsible for this translocation activity. A synthetic peptide consisting of the Tat basic amino acids 48-60 with a cysteine residue at the C-terminus coupled to fluorescein maleimide translocates to the cell nucleus as determined by fluorescence microscopy. In addition, a fusion protein (Tat-NLS- β -Gal) consisting of Tat amino acids 48-59 fused by their amino-terminus to β -galactosidase amino acids 9-1023 translocates to the cell nucleus in an ATP-dependent, cytosolic factor-independent manner. Accordingly, the fusion proteins of the present invention may comprise all or a portion of HIV-Tat, such as any sequential residues of the Tat protein basic peptide motif 37-72 (37-CFITKALGISYGRKKRRQRRRPPQGSQTHQVSLSKQ-72 (SEQ ID NO:56). The minimum number of amino acid residues can be in the range of from about three to about six. In one embodiment, the Tat portion of the fusion protein is from about three to about five contiguous amino acids in length. In another embodiment, the Tat portion of the fusion protein is about four amino acids in length, *i.e.*, the minimal requirement for one alpha helical turn. In another embodiment, the Tat portion of the fusion protein comprises Tat protein residues 48-57 (GRKKRRQRRR) (SEQ ID NO:57).

[0090] In additional embodiments of fusion proteins, a region may be added to facilitate purification. For example, “histidine tags” (“his tags”) or “lysine tags” (the second fusion peptide) may be appended to the first fusion peptide. Examples of histidine tags include, but are not limited to hexaH, heptaH and hexaHN. Examples of lysine tags include, but are not limited to pentaL, heptaL and FLAG. Such regions may be removed prior to final preparation of the protein. Other examples of a second fusion peptide include, but are not limited to, glutathione S-transferase (GST) and alkaline phosphatase (AP).

[0091] The addition of peptide moieties to proteins, whether to engender secretion or excretion, to improve stability and to facilitate purification or translocation, among others, is a familiar and routine technique in the art and may include modifying amino acids at the terminus to accommodate the tags. For example in any of SEQ ID NO: 16-35, the N-terminus amino acid may be modified to, for example, arginine and/or serine to accommodate a tag. Of course, the amino acid residues of the C-terminus may also be modified to accommodate tags. One particularly useful fusion protein comprises a heterologous region from immunoglobulin that can be used solubilize proteins. For example, EP A0464 533 discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thereby results, for example, in improved pharmacokinetic properties (EP A0232 262). On the other hand, for some uses, it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described.

[0092] The fusion proteins of the current invention can be recovered and purified from recombinant cell cultures by well-known methods including, but not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, *e.g.*, immobilized metal affinity chromatography (IMAC), hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography (“HPLC”) may also be employed for purification. Well-known techniques for refolding protein may be employed to regenerate active conformation when the fusion protein is denatured during isolation and/or purification.

[0093] Fusion proteins of the present invention include, but are not limited to, products of chemical synthetic procedures and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the fusion proteins of the present invention may be glycosylated or may be non-glycosylated. In addition, fusion proteins of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

[0094] The invention also relates to isolated nucleic acids and to constructs comprising these nucleic acids. The nucleic acids of the invention can be DNA or RNA, for example, mRNA. The nucleic acid molecules can be double-stranded or single-stranded; single stranded RNA or DNA can be the coding, or sense, strand or the non-coding, or antisense, strand. In particular, the nucleic acids may encode any polypeptide of the invention, including, but not limited to, the fusion proteins of the present invention. For example, the nucleic acids of the invention include polynucleotide sequences that encode glutathione-S-transferase (GST) fusion protein, poly-histidine (*e.g.*, His₆), poly-HN, poly-lysine, hemagglutinin, HSV-Tag and at least a portion of HIV-Tat. If desired, the nucleotide sequence of the isolated nucleic acid can include additional non-coding sequences such as non-coding 3' and 5' sequences (including regulatory sequences, for example).

[0095] In some embodiments, the isolated nucleic acid of the present invention further relates to the nucleic acids consisting of a nucleic acid sequence at least 95% identical to any of the nucleic acid sequences of SEQ ID NO: 58-77. In further embodiments, the invention therefore provides the nucleic acids consisting of a nucleic acid sequence at least about 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to any of the nucleic acid sequences of SEQ ID NO: 58-77.

[0096] The nucleic acid molecules of the invention can be "isolated." As used herein, an "isolated" nucleic acid molecule or nucleotide sequence is intended to mean a nucleic acid molecule or nucleotide sequence that is not flanked by nucleotide sequences normally flanking the gene or nucleotide sequence (as in genomic sequences) and/or has been completely or partially removed from its native environment (*e.g.*, a cell, tissue). For example, nucleic acid molecules that have been removed or purified from cells are considered isolated. In some instances, the isolated material will form part of a composition (for example, a crude extract containing other substances), buffer system or reagent mix. In other circumstances, the material may be purified to near homogeneity, for example as determined by PAGE or column chromatography such as HPLC. Thus, an isolated nucleic acid molecule or nucleotide sequence can include a nucleic acid molecule or nucleotide sequence which is synthesized chemically, using recombinant DNA technology or using any other suitable method. To be clear, a nucleic acid contained in a vector would be included in the definition of "isolated" as used herein. Also, isolated nucleotide sequences include recombinant nucleic acid molecules (*e.g.*, DNA, RNA) in heterologous organisms, as well as partially or substantially purified nucleic acids in solution. "Purified," on the other hand is well understood in the art and generally means that the nucleic acid molecules are substantially free of cellular material, cellular components, chemical precursors or other chemicals beyond, perhaps, buffer or solvent. "Substantially free" is not intended to mean that other components beyond the novel nucleic acid molecules are undetectable. The nucleic acid molecules of the present invention may be isolated or purified. Both *in vivo* and *in*

vitro RNA transcripts of a DNA molecule of the present invention are also encompassed by “isolated” nucleotide sequences.

[0097] The invention also encompasses variations of the nucleotide sequences of the invention, such as those encoding functional fragments or variants of the polypeptides as described above. Such variants can be naturally-occurring, or non-naturally-occurring, such as those induced by various mutagens and mutagenic processes. Intended variations include, but are not limited to, addition, deletion and substitution of one or more nucleotides which can result in conservative or non-conservative amino acid changes, including additions and deletions.

[0098] The invention described herein also relates to fragments of the isolated nucleic acid molecules described herein. The term “fragment” is intended to encompass a portion of a nucleotide sequence described herein which is from at least about 20 contiguous nucleotides to at least about 50 contiguous nucleotides or longer in length. Such fragments may be useful as probes and primers. In particular, primers and probes may selectively hybridize to the nucleic acid molecule encoding the polypeptides described herein. For example, fragments which encode polypeptides that retain activity, as described below, are particularly useful.

[0099] The invention also provides nucleic acid molecules that hybridize under high stringency hybridization conditions, such as for selective hybridization, to the nucleotide sequences described herein (*e.g.*, nucleic acid molecules which specifically hybridize to a nucleotide sequence encoding polypeptides described herein and encode a modified growth factor). Hybridization probes include synthetic oligonucleotides which bind in a base-specific manner to a complementary strand of nucleic acid.

[00100] Such nucleic acid molecules can be detected and/or isolated by specific hybridization *e.g.*, under high stringency conditions. “Stringency conditions” for hybridization is a term of art that refers to the incubation and wash conditions, *e.g.*, conditions of temperature and buffer concentration, which permit hybridization of a particular nucleic acid to a second nucleic acid; the first nucleic acid may be perfectly complementary, *i.e.*, 100%, to the second, or the first and second may share some degree of complementarity, which is less than perfect, *e.g.*, 60%, 75%, 85%, 95% or more. For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity.

[00101] “High stringency conditions,” “moderate stringency conditions” and “low stringency conditions” for nucleic acid hybridizations are explained in Current Protocols in Molecular Biology, John Wiley & Sons). The exact conditions which determine the stringency of hybridization depend not only on ionic strength, *e.g.*, 0.2× SSC, 0.1× SSC of the wash buffers, temperature, *e.g.*, room temperature, 42°C., 68°C., etc., and the concentration of destabilizing agents such as formamide or denaturing agents such as SDS, but also on factors such as the length of the nucleic acid sequence,

base composition, percent mismatch between hybridizing sequences and the frequency of occurrence of subsets of that sequence within other non-identical sequences. Thus, high, moderate or low stringency conditions may be determined empirically.

[00102] By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which will allow a given sequence to hybridize with the most similar sequences in the sample can be determined. Exemplary conditions are described in Krause (1991) *Methods in Enzymology*, 200:546-556. Washing is the step in which conditions are usually set so as to determine a minimum level of complementarity of the hybrids. Generally, starting from the lowest temperature at which only homologous hybridization occurs, each degree (°C) by which the final wash temperature is reduced, while holding SSC concentration constant, allows an increase by 1% in the maximum extent of mismatching among the sequences that hybridize. Generally, doubling the concentration of SSC results in an increase in T_m . Using these guidelines, the washing temperature can be determined empirically for high, moderate or low stringency, depending on the level of mismatch sought. Exemplary high stringency conditions include, but are not limited to, hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in $0.1 \times$ SSC at 60°C. Example of progressively higher stringency conditions include, after hybridization, washing with $0.2 \times$ SSC and 0.1% SDS at about room temperature (low stringency conditions); washing with $0.2 \times$ SSC, and 0.1% SDS at about 42°C (moderate stringency conditions); and washing with $0.1 \times$ SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, washing may encompass two or more of the stringency conditions in order of increasing stringency. Optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

[00103] Equivalent conditions can be determined by varying one or more of the parameters given as an example, as known in the art, while maintaining a similar degree of identity or similarity between the target nucleic acid molecule and the primer or probe used. Hybridizable nucleotide sequences are useful as probes and primers for identification of organisms comprising a nucleic acid of the invention and/or to isolate a nucleic acid of the invention, for example. The term “primer” is used herein as it is in the art and refers to a single-stranded oligonucleotide which acts as a point of initiation of template-directed DNA synthesis under appropriate conditions in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer, but typically ranges from about 15 to about 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template, but must be sufficiently complementary to hybridize with a template. The term “primer site” refers to the area of the target DNA to which a primer hybridizes. The term “primer pair” refers to a set of primers including a 5' (upstream) primer that

hybridizes with the 5' end of the DNA sequence to be amplified and a 3' (downstream) primer that hybridizes with the complement of the 3' end of the sequence to be amplified.

[00104] The present invention also relates to vectors that include nucleic acid molecules of the present invention, host cells that are genetically engineered with vectors of the invention and the production of proteins of the invention by recombinant techniques.

[00105] In accordance with this aspect of the invention, the vector may be, for example, a plasmid vector, a single-or double-stranded phage vector, or a single-or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, for example DNA, by well-known techniques for introducing DNA and RNA into cells. Viral vectors may be replication competent or replication defective. In the latter, case viral propagation generally will occur only in complementing host cells.

[00106] In certain respects, the vectors to be used are those for expression of polynucleotides and proteins of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

[00107] A great variety of expression vectors can be used to express the proteins of the invention. Such vectors include chromosomal, episomal and virus-derived vectors, *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as adeno-associated virus, lentivirus, baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. All may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides or proteins in a host may be used for expression in this regard.

[00108] The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s) including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include, but are not limited to, the phage lambda PL promoter, the E. coli lac, trp and tac promoters, HIV promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. In general, expression constructs will contain sites for transcription, initiation and termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

[00109] In addition, the constructs may contain control regions that regulate, as well as engender expression. Generally, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

[00110] Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors may contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline, kanamycin or ampicillin resistance genes for culturing *E. coli* and other bacteria.

[00111] The vector containing the appropriate DNA sequence, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well-known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as, but not limited to, *E. coli*, *Streptomyces*, *Bacillus*, and *Salmonella* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Hosts for a great variety of expression constructs are well known, and those of skill in the art will be enabled by the present disclosure to select an appropriate host for expressing one of the proteins of the present invention.

[00112] Examples of vectors that may be useful for fusion proteins include, but are not limited to, pGEX (Pharmacia), pMAL (New England Biolabs) and pRIT5 (Pharmacia) that fuse glutathione-S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase.

[00113] Examples of vectors for expression in yeast *S. cerevisiae* include pYepSecl (Baldari (1987) EMBO J. 6, 229-234), pMFa (Kurjan (1982) Cell 30, 933-943), pJRY88 (Schultz (1987) Gene 54, 137-123), pYES2 (Invitrogen) and picZ (Invitrogen).

[00114] Alternatively, the modified growth factors can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (*e.g.*, Sf9 cells) include the pAc series (Smith (1983) Mol. Cell. Biol. 3, 2156-2165) and the pVL series (Lucklow (1989) Virology 170, 31-39).

[00115] In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8

(Seed (1987) Nature 329, 840) and pMT2PC (Kaufman (1987) EMBO J. 6, 187 195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells, see Sambrook (2010) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

[00116] In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include liver-specific promoters (*e.g.*, albumin promoter), lymphoid-specific promoters such as, but not limited to, T cell receptors and immunoglobulins, neuron-specific promoters (*e.g.*, neurofilament promoter), pancreas-specific promoters, mammary gland-specific promoters (*e.g.*, milk whey promoter), bone-specific promoters (*e.g.*, osteocalcin, osteopontin or bone sialoprotein, promoter regions), cartilage specific promoters (*e.g.*, WARP) and muscle specific promoters (Desmin, myoglobin, etc) just to name a few. Developmentally-regulated promoters are also encompassed, *e.g.*, the murine hox promoters (Kessel and Gruss (1990) Science 249, 374 379) and the α -fetoprotein promoter (Campes and Tilghman (1989) Genes Dev. 3, 537 546).

[00117] The present invention also relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. The host cell can be stably or transiently transfected with the construct. The polynucleotides may be introduced alone or with other polynucleotides. Such other polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention. As used herein, a "host cell" is a cell that normally does not contain any of the nucleotides of the present invention and contains at least one copy of the nucleotides of the present invention. Thus, a host cell as used herein can be a cell in a culture setting or the host cell can be in an organism setting where the host cell is part of an organism, organ or tissue.

[00118] Suitable host cells for expression of the polypeptides of the invention include, but are not limited to, prokaryotes, yeast, and eukaryotes. If a prokaryotic expression vector is employed, then the appropriate host cell would be any prokaryotic cell capable of expressing the cloned sequences. Suitable prokaryotic cells include, but are not limited to, bacteria of the genera *Escherichia*, *Bacillus*, *Pseudomonas*, *Staphylococcus*, and *Streptomyces*.

[00119] If a eukaryotic expression vector is employed, then the appropriate host cell would be any eukaryotic cell capable of expressing the cloned sequence. In one embodiment, eukaryotic cells are

cells of higher eukaryotes. Suitable eukaryotic cells include, but are not limited to, non-human mammalian tissue culture cells and human tissue culture cells. Other host cells include, but are not limited to, insect cells, HeLa cells, Chinese hamster ovary cells (CHO cells), African green monkey kidney cells (COS cells), human 293 cells, and murine 3T3 fibroblasts.

[00120] In addition, a yeast cell may be employed as a host cell. Yeast cells include, but are not limited to, the genera *Saccharomyces*, *Pichia* and *Kluyveromyces*. In one embodiment, the yeast hosts are *S. cerevisiae* or *P. pastoris*. Yeast vectors may contain an origin of replication sequence from a 2T yeast plasmid, an autonomously replication sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination and a selectable marker gene. Shuttle vectors for replication in both yeast and *E. coli* are also included herein.

[00121] Introduction of a construct into the host cell can be affected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods.

[00122] For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. To identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Various selectable markers include those that confer resistance to drugs, such as G418, hygromycin, dihydrofolate reductase (DHFR) and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding modified growth factor or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die). In one embodiment, the polypeptides of the invention are expressed in Chinese Hamster Ovary (CHO) cells.

[00123] The modified growth factor expression vectors prepared as above are introduced into CHO cells by any known method, including, but not limited to the calcium phosphate method and electroporation.

[00124] Transformants carrying the expression vectors are selected based on the above-mentioned selectable markers. Repeated clonal selection of the transformants using the selectable markers allows selection of stable cell lines expressing the modified growth factor constructs. Increased concentrations in the selection medium allows gene amplification and greater expression of the desired modified growth factor. The host cells, for example CHO cells, containing the recombinant modified growth factor can be produced by cultivating the CHO cells containing the modified growth factor expression vectors constitutively expressing the modified growth factor constructs.

[00125] Accordingly, the current invention also relates to methods of producing a modified growth factor comprising culturing the host cells of the invention under conditions such that the modified growth factor is expressed, and recovering said protein. The culture conditions required to express the proteins of the current invention are dependent upon the host cells that are harboring the polynucleotides of the current invention. The culture conditions for each cell type are well-known in the art and can be easily optimized, if necessary. For example, a nucleic acid encoding a polypeptide of the invention, or a construct comprising such nucleic acid, can be introduced into a suitable host cell by a method appropriate to the host cell selected, *e.g.*, transformation, transfection, electroporation, infection, such that the nucleic acid is operably linked to one or more expression control elements as described herein. Host cells can be maintained under conditions suitable for expression in vitro or in vivo, whereby the encoded polypeptide is produced. For example host cells may be maintained in the presence of an inducer, suitable media supplemented with appropriate salts, growth factors, antibiotic, nutritional supplements, etc., which may facilitate protein expression. In additional embodiments, the modified growth factors of the invention can be produced by in vitro translation of a nucleic acid that encodes the modified growth factor, by chemical synthesis or by any other suitable method. If desired, the modified growth factor can be isolated from the host cell or other environment in which the protein is produced or secreted. It should therefore be appreciated that the methods of producing the modified growth factors encompass expression of the polypeptides in a host cell of a transgenic animal or plant. See U.S. Patent Nos. 6,013,857, 5,990,385, and 5,994,616.

[00126] In situations where the modified BMP will be found primarily intracellularly, intracellular material (including inclusion bodies for Gram-negative bacteria) can be extracted from the host cell using any standard technique known to one of ordinary skill in the art. Such methods would encompass, by way of example and not by way of limitation, lysing the host cells to release the contents of the periplasm/cytoplasm by French press, homogenization and/or sonication followed by centrifugation.

[00127] If the modified BMP has formed inclusion bodies in the cytosol, such inclusion bodies may frequently bind to the inner and/or outer cellular membranes. Upon centrifugation, the inclusion bodies will be found primarily in the pellet material. The pellet material can then be treated at pH extremes or with one or more chaotropic agents such as a detergent, guanidine, guanidine derivatives, urea, or urea derivatives in the presence of a reducing agent such as dithiothreitol at alkaline pH or tris-carboxyethyl phosphine at acid pH to release, break apart and solubilize the inclusion bodies. Once solubilized, the modified growth factor peptide can be analyzed using gel electrophoresis, immunoprecipitation or the like. Various methods of isolating the modified growth factor peptide would be apparent to one of ordinary skill in the art, for example, isolation may be accomplished

using standard methods such as those set forth below and in Marston et al (1990) Meth. Enzymol. 182, 264-275.

[00128] If the modified growth factor peptide is not biologically active following the isolation procedure employed, various methods for “refolding” or converting the polypeptide to its tertiary structure and generating disulfide linkages, can be used to restore biological activity. Methods known to one of ordinary skill in the art include adjusting the pH of the solubilized polypeptide to a certain pH, usually above 7, and in the presence of a particular concentration of a chaotrope. The selection of chaotrope is very similar to the choices used for inclusion body solubilization, but usually at a lower concentration, and is not necessarily the same chaotrope as used for the solubilization. It may be required to employ a reducing agent or the reducing agent plus its oxidized form in a specific ratio, to generate a particular redox potential allowing for disulfide shuffling during the formation of the protein’s cysteine bridge(s). Some of the commonly used redox couples include cysteine/cysteine, glutathione (GSH)/dithiobis GSH, cupric chloride, dithiothreitol (DTT)/dithiane DTT, 2-mercaptoethanol (bME)/dithio-b(ME). To increase the efficiency of the refolding, it may be necessary to employ a cosolvent, such as glycerol, polyethylene glycol of various molecular weights and arginine.

[00129] Other methods of preparing the modified growth factors of the present invention include, but are not limited to, contacting a form of BMP with a protease. In one embodiment, the methods comprise contacting the mature form of BMP with a protease to produce the modified growth factors of the present invention.

[00130] As used herein, the term “protease” refers to any of various enzymes that catalyze the hydrolytic breakdown of proteins into smaller segments, including polypeptides and/or amino acids. Accordingly, included in the definition are proteinases and peptidases. The term “proteinase” refers to a protease that catalyzes the hydrolytic breakdown of proteins by splitting them into smaller peptide fractions, *i.e.*, polypeptides or amino acids. The term “peptidase” refers to a protease that catalyzes the hydrolytic breakdown of peptides via cleavage of an individual amino acid from the peptide chain.

[00131] In one embodiment, the protease is a serine protease, a threonine protease, a metalloproteinase, a cysteine protease, an aspartate protease or a glutamic acid protease. Examples of such proteases are well known in the art and include proteases from both eukaryotic and prokaryotic sources. Examples of serine proteases include, but are not limited to, chymotrypsin, trypsin, thrombin, elastase, subtilisin and alpha/beta hydrolases. Examples of cysteine proteases include, but are not limited to, actinidain, bromelain, calpains, some cathepsins, clostripain and papain. Examples of aspartate proteases include, but are not limited to, some cathepsins, chyomsin, renin, pepsin and HIV-1 protease. Examples of metalloproteinases include, but are not limited to, the matrix

metalloproteinase (MMP) family of proteases that include MMP-1, MMP-2, MMP-3, MMP-4, MMP-5, MMP-6, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-15, MMP-16, MMP-17, MMP-18 and membrane-type MMP (MT-MMP), to name a few. Many of these MMPs are known by other names in the art and one of skill will be informed of synonyms of the various enzymes. For example, some of the “collagenases” are MMP-1, MMP-8, MMP-13, MMP-14, MMP-18 and dispaes, and some of the “gelatinases” are MMP-2, MMP-9 and MMP-12; some of the “stromelysins” are MMP-3, MMP-10 and MMP-11, and some of the membrane-type matrix metalloproteinases are MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25.

Collagenases are examples of metalloproteinases that break down native collagen or gelatin. A variety of microorganisms and many different types of animal cells produce collagenases. Bacterial collagenases are often used in the laboratory to digest tissues and isolate individual cells. Collagenase produced by *Clostridium histolyticum* (*C. histolyticum*) is a zinc metalloproteinase that degrades various types of collagen and gelatin. Additional examples of metalloproteinases are the astacin family, such as, but not limited to, astacin, tolloid, xolloid, mammalian tolloid like (mTLL), BMP-1, meprin A and B, and matrilysin and the aggrecanase (ADAMTS) family of metalloproteinase, such as, but not limited to, ADAMTS-1, ADAMTS-2, ADAMTS-3, ADAMTS-4, ADAMTS-5.

[00132] Of course, the methods include contacting a form of BMP with more than one protease either sequentially or simultaneously. In addition, the proteases used herein can be recombinant or isolated from various sources. The proteases used herein need not be isolated from the same cellular or animal source as the mature form of the BMP. For example, many proteases are found only in prokaryotes, yet these proteases are functional against proteins from eukaryotes. Factors affecting enzyme activity include, but are not limited to, salt concentrations of buffer, pH and temperature. One of skill in the art will readily understand the conditions necessary to promote peptide cleave based upon the enzyme used.

[00133] In some embodiments, the ratio of the amount of protease(s) to the amount of growth factor(s) may be between about 1 mol protease:1000 mol growth factor and about 1000 mol:1 mol, between about 1 mol protease:1000 mol growth factor and about 100 mol:1 mol, between about 1 mol:100 mol and about 100 mol:1 mol, between about 1 mol:100 mol and about 10 mol:1 mol, between about 1 mol:10 mol and about 10 mol:1 mol, or between about 1 mol:10 mol and about 1 mol:1 mol. In further embodiments, the ratio of the amount of collagenase(s) to the amount of growth factor(s) may be between about 1 mol collagenase:100 mol growth factor and about 1000 mol:1 mol, between about 1 mol:10 mol and about 1000 mol:1 mol, between about 1 mol:10 mol and about 100 mol:1 mol, or between about 1 mol:1 mol and about 10 mol:1 mol. The ratio of the amount of trypsin to the amount of growth factor(s) may be between about 1 mol trypsin: 1000 mol growth factor and about 1000 mol:1 mol, between about 1 mol:1000 mol and about 100 mol:1 mol, between about 1 mol:100 mol

and about 10 mol:1 mol, or between about 1 mol:10 mol and about 10 mol:1 mol. The ratio of the amount of clostripain to the amount of growth factor(s) may be between about 1 mol clostripain:1000 mol growth factor and about 1000 mol:1 mol, between about 1 mol:100 mol and about 1000 mol:1 mol, between about 1 mol:10 mol and about 100 mol:1 mol, or between about 1 mol:1 mol and about 10 mol:1 mol. The ratio of the amount of dispase to the amount of growth factor(s) may be between about 1 mol dispase: 1000 mol growth factor and about 1000 mol:1 mol, between about 1 mol:1000 mol and about 100 mol:1 mol, between about 1 mol:100 mol and about 10 mol:1 mol, or between about 1 mol:10 mol and about 10 mol:1 mol.

[00134] The treatment of growth factor(s) with proteases may be performed at a temperature between about 0°C and about 40°C, about 4°C and about 40°C, or at about 37°C or lower, in some embodiments. A growth factor may be treated with collagenase for between about 30 minutes and 3 days, about 1 hour and about 48 hours, about 2 hours and about 48 hours, or about 2 hours and about 24 hours, in certain embodiments. A growth factor may be treated with trypsin or clostripain for between about 5 minutes and 3 days, about 5 minutes and about 48 hours, about 5 minutes and about 24 hours, or about 5 minutes and about 8 hours. A growth factor may be treated with dispase for between about 5 minutes and 2 days, about 5 minutes and about 24 hours, about 5 minutes and about 18 hours, or about 5 minutes and about 8 hours. All these conditions do not count the extra protein or peptide substrate in the treatment mixture. Treatment may include mixing and/or incubation. Incubation may be performed under static or dynamic conditions, such as with agitation, shaking, stirring, mixing, horizontal motion, rocking, and others.

[00135] In some embodiments, the protease may be a recombinant protein or peptide fragment, a chemically synthesized protein or peptide fragment, or it may be extracted from a natural source and, optionally, modified, for example by being cleaved, inactivated, chemically-modified, or other methods. In other embodiments, certain aspects of the enzymatic activity of a protease may be inhibited or altered, and the protease having altered activity may be added to one or more growth factors to change the biological activity of the growth factor.

[00136] In additional embodiments, the activity of a protease may be modulated before, during, or after treatment of at least one growth factor with the protease. For example, the protease activity of a collagenase may be significantly reduced before it is used to treat a growth factor. In another example, the activity of a protease may be modulated before, during, or after administering to cells. In certain embodiments, the activity of a protease may be modulated by methods comprising heat inactivation, radiation inactivation, protease substrate neutralization, or chemical inhibition, among others. The chemical inhibitor used to modulate the activity of metalloproteinases may be selected from metal chelating agents, such as EDTA; cysteine and serine protease inhibitors, such as N-ethylmaleimide, phenylmethylsulfonyl fluoride (PMSF), and leupeptin; classical metalloproteinase inhibitor, such as

phosphoramidon, and bestatin; general protein inhibitor, such as α 2-macroglobulin, or natural or synthetic tissue inhibitors of metalloproteinases (TIMPs), such as TIMP-1, TIMP-2, TIMP-3, TIMP-4; and other inhibitors.

[00137] The form of BMP used in these methods can also be recombinant or can be isolated from a variety of cellular or animal sources. In one embodiment of the present invention, the BMP, in any form, that is contacted with the at least one protease is a recombinant BMP. In one specific embodiment, the recombinant BMP is contacted with a metalloproteinase to generate the modified BMP peptides of the present invention. In a more specific embodiment, the recombinant BMP is contacted with a collagenase to generate the novel, truncated BMP peptides of the present invention. In an even more specific embodiment, the recombinant BMP that is contacted with a collagenase is a recombinant mature form of BMP. In a still more specific embodiment, recombinant mature BMP is contacted with a collagenase and/or clostripain to generate the novel, truncated BMP peptides of the present invention. In another specific embodiment, recombinant mature BMP is contacted with a trypsin or dispase to generate the novel, truncated BMP peptides of the present invention.

[00138] Once treated with at least one protease, in some embodiments, the resulting peptides can be isolated and purified using routine methods in the art. Examples of purification methods include, but are not limited to size exclusion chromatography, high-performance liquid chromatography, ion exchange chromatography, electrophoresis, Western blotting and subsequent processing of the membrane. In one embodiment of the present invention, the resulting peptide that is purified after protease treatment comprises any of the amino acid sequence of SEQ ID NO:16-35. In other embodiments, after being treated with a protease, the resulting peptides are not isolated or purified and are administered to a cell in the presence of the protease. In another embodiment, the resulting peptide is isolated or purified and then is administered to a cell with a protease. In again another embodiment, after being treated with a protease, without being isolated or purified, the resulting peptides are administered to a cell in the presence of the protease, then another protease is administered together.

[00139] The modified growth factor peptides of the present invention may also be prepared by synthetic methods using solid-phase synthetic techniques. The synthesized polypeptides may be re-natured into correct folding pattern under appropriate conditions and bioactive dimers can be induced from monomers using appropriate buffers and conditions. The correctly folded dimers maybe further separated from monomers using appropriate column to increase the yield of functional bioactive factors.

[00140] Regardless of method of preparation, be it recombinantly, enzymatically or synthetically, the novel modified growth factors, as monomers or dimers, can be prepared as a composition. In one embodiment, the composition is a pharmaceutical composition. For example, one or more cofactors

may be added to the truncated mature bioactive factor of the present invention to form a composition. Cofactors that may be added include, but are not limited to, heparin, hyaluronic acid, a fibronectin, an elastin, a laminin, albumin, a proteoglycan, collagen, gelatin, a divalent cation, calcium chloride, zinc sulfate, magnesium chloride, sodium bicarbonate, sodium chloride, sodium acetate, or sodium phosphate. In some embodiments, a protein or a protein fragment may be added as a cofactor to the modified growth factor peptides of the present invention.

[00141] The compositions, or pharmaceutical compositions, comprising the nucleic acid molecules or polypeptides typically comprise the nucleic acid molecule or protein and a pharmaceutically acceptable carrier. As used herein, “pharmaceutically acceptable carrier” or “pharmaceutical carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The nature of the pharmaceutical carrier or other ingredients will depend on the specific route of administration and particular embodiment of the invention to be administered. Examples of techniques and protocols that are useful in this context are, inter alia, found in Remington: The Science and Practice of Pharmacy (2010), Lippincott, Williams & Wilkins. Examples of such pharmaceutical carriers or diluents include, but are not limited to, water, saline, Ringer’s solution, dextrose solution and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[00142] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include oral and parenteral (*e.g.*, intravenous, intradermal, subcutaneous, inhalation, transdermal (topical), transmucosal and rectal administration). Solutions or suspensions used for parenteral, intradermal or subcutaneous application can include, but are not limited to, a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents, antibacterial agents such as benzyl alcohol or methyl parabens, antioxidants such as ascorbic acid or sodium bisulfite, chelating agents such as ethylenediaminetetraacetic acid, buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00143] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile

injectable solutions or dispersion. For intravenous administration, suitable pharmaceutical carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF) or phosphate buffered saline (PBS). In all cases, the compositions must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The pharmaceutical carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it may be desirable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[00144] Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, the modified growth factor) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[00145] Oral compositions generally include an inert diluent or an edible pharmaceutical carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches or capsules. Oral compositions can also be prepared using a fluid pharmaceutical carrier for use as a mouthwash, wherein the compound in the fluid pharmaceutical carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like may contain any of the following ingredients, or compounds of a similar nature, such as but not limited to a binder, such as microcrystalline cellulose, gum tragacanth or gelatin, an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel or corn starch, a lubricant such as magnesium stearate or Sterotes, a glidant such as colloidal silicon dioxide, a sweetening agent such as sucrose or saccharin, or a flavoring agent such as peppermint, methyl salicylate or orange flavoring.

[00146] In one embodiment, the active compounds are prepared with pharmaceutical carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These compositions can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811. It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved.

[00147] The pharmaceutical compositions can be included in a container, pack or dispenser together with instructions for administration.

[00148] The dosage of BMP peptides or the composition comprising the BMP peptides and a protease will depend on the disease state or condition to be treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating human or animals, between approximately 0.005 mg/kg of body weight to 500 mg/kg of body weight of the compound can be administered. Therapy is typically administered at lower dosages and is continued until the desired therapeutic outcome is observed.

[00149] Methods of determining the dosages of compounds to be administered to a patient and modes of administering compounds to an organism are disclosed in, for example, WO 96/22976. Those skilled in the art will appreciate that such descriptions are applicable to the present invention and can be easily adapted to it.

[00150] The proper dosage depends on various factors such as the type of disease being treated, the particular composition being used and the size and physiological condition of the patient. Therapeutically effective doses for the compounds described herein can be estimated initially from cell culture and animal models. For example, a dose can be formulated in animal models to achieve a circulating concentration range that initially takes into account the IC_{50} as determined in cell culture assays. The animal model data can be used to more accurately determine useful doses in humans.

[00151] The invention also relates to methods of altering intracellular signaling of a cell, comprising contacting the cells with at least one of the modified peptides or the composition comprising the modified peptide and a protease, wherein the cell possesses a receptor that specifically binds to the modified growth factors of the present invention. The peptides utilized in the methods can be in monomeric, homodimeric or heterodimeric form. The specific binding of the modified growth factors to its receptor will, in turn, initiate the intracellular signaling cascade that is normally associated with the unmodified forms of the growth factor. For example, the “mature” form of BMP normally binds to its receptor complex including type I and type II receptors. The receptor phosphorylates cytoplasmic targets that include, but is not limited to, the Smad family of proteins. Smads are a class of proteins that function as intracellular signaling effectors for the TGF- β superfamily of secreted polypeptides. The activated BMP type I receptors will phosphorylate Smad1, Smad 5, and/or Smad 8. Phosphorylated Smad 1, 5, and 8 proteins, in turn, form a complex with Smad 4 and then translocate into the nucleus and interact with the transcription factors that regulate the expression of target genes.

[00152] Accordingly, the present invention provides methods of stimulating phosphorylation of Smad 1, Smad 5 and/or Smad 8 in a cell comprising contacting the cell(s) with at least one modified growth factor of the invention. The peptides utilized in the methods can be in monomeric, homodimeric or heterodimeric form. The activity of the novel peptides with respect to phosphorylating the Smad proteins may or may not be altered relative to the normal, mature growth factor. For example, the novel modified growth factors may increase or decrease phosphorylation of Smad 1, 5 and/or 8 compared to receptor binding of normal, mature growth factor. As another example, the novel modified growth factors may bind tighter or looser to its receptor (lower K_d) compared to receptor binding of normal, mature growth factor. One of skill in the art can readily determine if a particular protein is more or less phosphorylated over control groups using well known techniques such as transcription of reporter genes, ELISA assays, etc. Additional methods of the present invention comprise assessing the levels of Smad phosphorylation, for example, Smad 1, Smad 5 or Smad 8, both before and after contacting the cell(s) with the novel peptides of the present invention and determining the increase or decrease of Smad phosphorylation in response to the novel peptides of the present invention.

[00153] In another embodiment, the present invention provides methods of stimulating promoter activity in a cell or population of cells, where the promoter is responsive to activated Smad complexes, with the methods comprising contacting the cell(s) with at least one modified growth factor peptide of the present invention. The peptides utilized in the methods can be in monomeric, homodimeric or heterodimeric form. One of skill in the art would be aware of promoters that respond to activated Smad complexes. See, for example, Massague (2000) Cell, 103:295-309. The activity of the novel peptides with respect to stimulating Smad-responsive promoters may or may not be altered relative to the normal, mature growth factor. For example, the novel modified growth factors may

increase or decrease activation of Smad-responsive promoters compared to receptor binding of normal, mature growth factor. One of skill in the art can readily determine if a promoter is more or less activated over control groups using well known techniques such as transcription of reporter genes, ELISA assays, etc. Additional methods of the present invention comprise assessing the activity of a Smad-responsive promoter both before and after contacting the cell(s) with the novel peptides of the present invention and determining the increase or decrease of the promoter in response to the novel peptides of the present invention.

[00154] As used herein, “contacting,” when used in connection with the methods of the present invention means bringing the novel peptides, in monomeric, homodimeric or heterodimeric form, in proximity to the target cells such that a specific binding event or a biological effect is possible. Thus, contacting can include adding the novel peptides in culture medium and applying the culture medium to cells in culture. Contacting also encompasses transfecting a cell with at least one vector described herein and allowing the cell to produce the modified growth factor. Of course, contacting would also include administration of the modified growth factor peptides, or pharmaceutical compositions thereof, of the present invention to cells in an intact organism. Compositions for administering the novel peptides of the present invention have been described herein.

[00155] As used herein, “administering,” and “administer” are used to mean introducing at least one compound comprising at least one novel modified growth factor peptide into a subject. The peptides utilized in the administration methods can be in monomeric, homodimeric or heterodimeric form. When administration is for the purpose of treatment, the substance is provided at, or after the onset of, a symptom or condition in need of treatment. The therapeutic administration of this substance serves to attenuate any symptom, or prevent additional symptoms from arising. When administration is for the purposes of preventing a condition from arising (“prophylactic administration”), the substance is provided in advance of any visible or detectable symptom. The prophylactic administration of the substance serves to attenuate subsequently arising symptoms or prevent symptoms from arising altogether. The route of administration of the compound includes, but is not limited to, topical, transdermal, intranasal, vaginal, rectal, oral, subcutaneous intravenous, intraarterial, intramuscular, intraosseous, intraperitoneal, epidural and intrathecal as previously disclosed herein.

[00156] Furthermore, the methods would also include coadministering one or more substances in addition to the novel peptides of the present invention. The term “coadminister” indicates that each of at least two compounds is administered during a time frame wherein the respective periods of biological activity or effects overlap. Thus the term includes sequential as well as coextensive administration of the compounds of the present invention. And similar to administering compounds, coadministration of more than one substance can be for therapeutic and/or prophylactic purposes. If

more than one substance is coadministered, the routes of administration of the two or more substances need not be the same.

[00157] The invention also relates to methods of promoting osteoinductivity, with the methods comprising contacting cells with at least one modified growth factor peptide of the present invention, or compositions described herein. As used herein, “osteoinductivity” can refer to causing cells to differentiate into cells that are more osteoblast-like in phenotype, or the term can refer to increasing the proliferation of osteoblasts, or both. The cells, prior to contact with the modified growth factor peptide(s) of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The osteoinductive activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor.

[00158] The invention also relates to methods of promoting chondroinductivity, with the methods comprising contacting cells with at least one modified growth factor peptide of the present invention, or compositions described herein. As used herein, “chondroinductivity” can refer to causing cells to differentiate into cells that are more chondrocyte-like in phenotype, or the term can refer to increasing the proliferation of chondrocytes, or both. The cells, prior to contact with the modified growth factor peptide(s) of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The chondroinductive activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor.

[00159] The invention also relates to methods of promoting ligament/tendon differentiation, with the methods comprising contacting cells with at least one modified growth factor peptide of the present invention, or compositions described herein. As used herein, “ligament/tendon differentiation” can refer to causing cells to differentiate into cells that are more ligament and/or tendon-like in phenotype, or the term can refer to increasing the proliferation of ligament and/or tendon, or both. The cells, prior to contact with the modified growth factor peptide(s) of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The ligament/tendon differentiation activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor.

[00160] For example, the invention provides for growing and/or culturing cells in the presence of one or more modified growth factor peptides, or compositions described herein. “Growing and/or culturing cells in the presence of” includes traditional cell culture methods as well as placing cells in the presence of the modified growth factors in any setting, such as in natural or synthetic matrices or tissues. The cells may be mammalian, such as but not limited to human, bovine, porcine, murine,

ovine, equine, canine, feline and others. In some embodiments, the cells may be mesenchymal stem cells, such as adipose-derived stem cells, embryonic stem cells, progenitor cells, differentiated cells, undifferentiated cells, and/or pluripotent stem cells. Appropriate cells may also include, but are not limited to cells of the ectodermal lineage, cells of the mesodermal lineage, and cells of the endodermal lineage. Examples of cells of the ectodermal lineage include but are not limited to keratinocytes, osteoblasts, chondrocytes, neurons. Examples of cells of the mesodermal lineage include but are not limited to myoblasts, adipocytes, fibroblasts, endothelial cells, or stromal cells. Examples of cells of the endodermal lineage include but not limited to epithelial cells of the auditory tube, the respiratory tract, such as trachea, bronchi, and alveoli of the lungs, the gastrointestinal tract, the urinary bladder and epithelial cells lining all glands. The cells may also be primary cells derived from tissues or organs. Appropriate cell lines used in the present invention may include but are not limited to mesenchymal cell lines, preosteoblastic cell lines, osteoblastic cell lines, and chondroblastic cell lines. The cells to which the modified growth factor peptides have been administered may be placed directly into a tissue, organism or other setting such as a matrix, including, but not limited to, bone matrices.

[00161] In some embodiments, the cells may be derived from autologous or allogeneic sources. The cells may be differentiated cells including chondrocytes, osteoblasts, osteoclasts, endothelial cells, epithelial cells, fibroblasts, and periosteal cells. Additionally, the cells may be totipotent, pluripotent, multipotent, progenitor, or adult somatic stem cells. The stem cells may be derived from embryos, placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, muscle. The stem cells may be derived from skeletal muscle, smooth muscle, and cardiac muscle. The stem cells may be derived from genetic reprogramming of mature cells, such as induced pluripotent stem cells (iPSCs).

[00162] Any cell described herewith may be cultured with one or more modified growth factors or compositions described herein for between about 15 minutes and about 4 weeks, about 2 hours and about 2 weeks, about 2 hours and about 1 week, about 2 hours and about 72 hours, about 24 hours and about 72 hours, or about 24 hours and about 96 hours, at between about 20°C and about 40°C or about 30°C and about 37°C, in an atmosphere containing between about 1% CO₂ and about 10% CO₂ or about 4% CO₂ and about 6% CO₂, in certain embodiments. In some embodiments of the present invention, cells may be cultured in the presence of one or more modified growth factors and/or (1) a tissue or an organ, (2) a matrix, or (3) a combination thereof. Cells that have been cultured in the presence of one or more modified growth factors in a cell culture medium may subsequently be applied to a matrix, a tissue, an organ or a combination thereof, in certain embodiments.

[00163] In some embodiments, treated cells are cryopreserved. Cryopreservation agents may be used to preserve treated cells, in certain embodiments. In some embodiments, treated cells are seeded onto

a matrix, and the cell-seeded matrix may be preserved using at least one cryopreservation agent. At least one cofactor may be added to treated cells, in some embodiments. Cofactors that may optionally be used are heparin, hyaluronic acid, a fibronectin, an elastin, a laminin, a proteoglycan, collagen, or gelatin, among others. In certain embodiments, a divalent cation, calcium chloride, zinc sulfate, magnesium chloride, sodium bicarbonate, sodium chloride, or sodium phosphate may be added as a cofactor to treated cells. In some embodiments, a protein or a protein fragment may be a cofactor that is added to treated cells. In further embodiments, a protease described herein may be a cofactor that is added to treated cells. Alternatively, the treated cells can be kept at a low temperature (*e.g.* 4 °C) or room temperature for 24-48 hours without cryopreservation.

[00164] In certain embodiments, treated cells that have been cryopreserved are optionally revived at a temperature between about 10°C and about 37°C before being applied to a tissue or an organ defect, a tissue, an organ, a matrix, or a mixture of two or more of these. In certain embodiments, treated cells may be combined with one or more body fluid, for example blood, platelet-rich plasma, platelet-poor plasma, plasma, bone marrow, and cord blood, among others, and an isotonic, hypotonic, or hypertonic solution, for instance saline, or Lactated ringer solution, and others, before they are used to treat a tissue or an organ defect. In some embodiments, treated cells may be applied to a tissue or an organ defect by injecting or inserting the cells between tissues or organs, or placing the cells on top of the defect. Modified growth factors may be administered to cells *in vitro*, *in vivo*, or *in situ*, in some embodiments. The modified growth factors may be administered to cells that are in tissue or organ or that have been isolated from tissues or organs, in some embodiments. In certain embodiments, at least one modified growth factor or compositions described herein may be administered to cells in (1) a cell culture medium, (2) a tissue or an organ, (3) a matrix, or (4) a combination of two or more of these.

[00165] There are a variety of osteoblast, chondrocyte, ligament/tendon differentiation markers that can be measured to assess osteoinductivity, chondroinductivity, or ligament/tendon differentiation, respectively. For example, cells express alkaline phosphatases during the early stages of differentiation toward osteoblast lineages. Therefore, *in vitro* alkaline phosphatase assays may be used to evaluate osteoinductivity in cells contacted with the modified growth factor peptide(s) of the present invention. The ability of the modified growth factor peptide(s) of the present invention to stimulate or induce the alkaline phosphatase expression in an otherwise non-bone forming cells, such as myoblast (C2C12 cells), would indicate that the modified growth factor peptide(s) of the present invention has osteoinductive activity. In these assays, cells cultured without added growth factors of any kind and without added modified growth factor peptide(s) of the present invention are used as negative controls to show that the baseline alkaline phosphatase expression on non-bone forming cells. The baseline of the osteoblastic markers in the negative control need not be zero, meaning that the cells in the negative control group may have at least some level of phenotypic marker(s). Accordingly, an “osteoinductive” peptide of the present invention would simply cause an increase in

the osteoblastic markers in experimental cells over control. Similarly, chondrocyte markers, including but not limited to type X collagen, type II collagen, Sox 9, Aggrecan, Matrilin-1 and CEP-68, to name a few, can be used to assess chondroinductive potential. Moreover, ligament/tendon markers, including but not limited to scleraxis, can be used to assess ligament/tendon differentiation potential.

[00166] Moreover, osteoinductivity, chondroinductivity, and ligament/tendon differentiation may be determined in tissue culture by investigating the ability of the modified growth factor peptide(s) of the present invention to differentiate or induce osteoblast phenotype, chondrocyte phenotype, ligament/tendon cell phenotype in cultured cells, such as primary cells, cell lines, or explants. For example, the cells may display increased production of a marker characteristic of osteoblasts and/or chondrocytes, such as alkaline phosphatase, etc. For example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the modified growth factors may be more than 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 times greater than those of the unmodified growth factors. In another example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the modified growth factors may be more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500 or even 1000 times greater compared to those of the unmodified growth factors. Of course, this indicates that lower concentrations of modified growth factor, compared to unmodified growth factor are required to achieve the same effects.

[00167] Osteoinductivity, chondroinductivity, ligament/tendon differentiation, for assessing the bone, cartilage, ligament or tendon forming potential induced by the modified growth factor peptide(s) of the present invention in a location such as muscle, may also be evaluated using a suitable animal model. For example, intramuscular implantation into a rodent has been used as a model to assess osteoinductive activity of bioactive factors.

[00168] The invention also relates to methods of promoting proliferation or maintaining the differentiated state or preventing de-differentiation of osteoblasts, chondrocytes, ligament cells, tendon cells and/or any cell type disclosed herein comprising administering to the osteoblasts, chondrocytes, ligament cells, or tendon cells the modified growth factor peptides or compositions described herein. The proliferative activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor.

[00169] Mitogenicity may be assessed by investigating cell proliferation induced by the modified growth factor peptides using various in vitro assays that measure metabolic activity, such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, alamarBlue[®] assay, and others. The alamarBlue[®] assay uses a non-cytotoxic reduction-oxidation indicator to measure cell metabolic activity, making it a nondestructive assay for assessing the mitogenic activity of the modified growth factors. Proliferation can also be assessed by measuring DNA quantification, such as by using a PicoGreen[™] DNA assay, radioactive labeling of DNA synthesis, such as [³H]thymidine labeling or

BrdU incorporation. Proliferation can also be assessed via manual cell counting, such as using a trypan blue hemacytometer.

[00170] The invention also relates to methods of increasing a cellular growth factor activity comprising administering to the cell at least one protease and the growth factor described herein. The protease is a protease described herein, including, but not limited to, collagenase, clostripain, dispase, trypsin, cathepsin, MMP-1, MMP-13, and a mixture thereof. In some embodiments, the growth factor comprises the mature growth factor and/or the modified growth factor described herein. The cell is a cell described herein, including, but not limited to, differentiated cells, adult stem cells, progenitor cells, and pluripotent stem cells. As explained previously, the modification of the growth factors as disclosed herein also enhances the activity of BMP. In certain embodiments, administering the protease along with the modified growth factor described herein further enhances the activity of the modified growth factor, such as an osteoinductive, chondroinductive activity, ligament/tendon differentiation potential.

[00171] In some embodiments, the addition of the protease described herein into the composition enhances stability of the growth factor in the same composition. As described previously, the modification of the growth factors as disclosed herein also enhances the stability of the growth factor. In certain embodiments, the addition of the protease into the composition comprising the modified growth factor further enhances the stability of the modified growth factor.

[00172] In additional embodiments, the composition administered to a cell in increasing a cellular growth factor activity comprises two or more protease. In some embodiments, a second protease can be mixed into the composition comprising the protease and the growth factor or can be administered separately from the composition comprising the protease and the growth factor.

[00173] The invention further relates to tissue or organ repair or regeneration compositions comprising (i) at least one modified growth factor peptide or the compositions of the present invention and (ii) a tissue, an organ, a matrix and/or a mixture of two or more thereof. The tissue or organ repair composition may comprise –bone, cartilage, and/or connective tissue.

[00174] An implantable biocompatible matrix for use with the compositions described herein can function as a suitable delivery or support system for the modified growth factor peptides. A biocompatible matrix should be non-toxic, non-eliciting or stimulating severe inflammatory response or immunological rejections, and devoid of other undesired reactions at the implantation site. Suitable matrices may also provide for release of the modified growth factor peptides, for example, to promote a slow, sustained release over time at the implantation site. In one embodiment, the matrix is a bone matrix or cartilage or connective tissue. In addition to its common, ordinary meaning, “administering to the matrix” includes embedding into the matrix or matrices host cells capable of producing at least one modified growth factors peptide. In another embodiment, the invention provides for methods of

treating cells with at least one modified growth factor peptide or compositions described herein and implanting these treated cells into a matrix, such as, but not limited to a bone matrix or cartilage.

[00175] Suitable matrices include, but are not limited to, porous scaffolds into which bone cells or progenitor cells may migrate. Osteogenic or chondrogenic cells, *i.e.*, cells involved in the process of deposition of new bone material or cartilaginous material, respectively, can often attach to such porous matrices, which can then serve as scaffolding for bone and cartilage tissue growth. Cells involved in the process of deposition of new ligament or tendon material can also attach to such porous matrices. For certain applications, the matrix should have sufficient mechanical strength to maintain its three dimensional structure and help support the immobilization of the bone segments being united or grafted together. Porous matrices which provide scaffolding for tissue growth can accelerate the deposition of new bone or the rate of bone growth and are said to be “osteochonductive.” Osteochonductive matrices are especially useful in the pharmaceutical compositions described herein. Porous matrices which provide scaffolding for tissue growth can accelerate the deposition of new cartilage or the rate of cartilage growth and are said to be “chondroconductive.” Osteochonductive matrices are especially useful in the pharmaceutical compositions described herein.

Chondroconductive matrices are especially useful in the pharmaceutical compositions described herein. The osteoinductive or chondroinductive activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor. Thus, the osteochonductive or chondroconductive activity of the treated matrices of the present invention may be enhanced compared to matrices not treated with modified growth factor. Of course, the matrices are considered to be osteochonductive or chondroconductive if cells within the matrix begin to differentiate into more osteoblast-like or chondrocyte-like appearing or functional cells, respectively.

[00176] Matrices can be derived from natural sources or they can be synthetic or a mixture of both. Matrices from natural sources may also comprise natural polymers, including, but not limited to, collagen, hyaluronic acid, alginate, albumin, fibrinogen-fibrin, chitosan, elasin, laminin, connective tissues, intervertebral disc, cortical or cancellous bone, demineralized or mineralized bone, fascia lata, dermis, muscle, ligament, tendon, cartilage including articular/hyaline cartilage, elastic cartilage, and fibrocartilage, a mixture thereof, and mixture of reconstituted tissue. Matrices from synthetic sources refer to any material not produced by living organisms, which may include, not limited to, the synthetic material made up of organic components, inorganic components, or a mixture thereof. In some embodiments, a synthetic matrix may comprise an organic synthetic polymer, such as poly(lactic-co-glycolic acid), polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA), polyhydroxybutyrate (PHB), Poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO)), and others. In some embodiments, a tissue, an organ, or matrix comprising at least one of alginate, chitosan, collagen, gelatin, hyaluronic acid, a fibronectin, an elastin, a laminin, and a proteoglycan

may be employed. In certain embodiments, a matrix comprising inorganic components, such as hydroxyapatite, calcium sulfate, octacalcium phosphate, calcium phosphate, macroporous calcium metaphosphate ceramic, β -tricalcium phosphate, metal, metal alloy, and others, may be used. A matrix used in certain embodiments of the present invention may be prepared by demineralizing, decellularizing or devitalizing a tissue or an organ and cells may be seeded onto the matrix.

[00177] In some embodiments, at least one modified growth factor compositions described herein may be applied to the matrix and may be incubated at conditions permitting the generation of a treated matrix. In some embodiments, incubation may be carried out at about 40°C or lower, or between about 10°C and about 37°C, or about 20°C and about 37°C. Incubation may be carried out for between at least about 2 minutes and about 120 minutes, about 3 minutes and about 100 minutes, about 4 minutes and about 80 minutes, about 5 minutes and about 60 minutes, and about 5 minutes and about 30 minutes in certain embodiments. Incubation may be performed under static or dynamic conditions, such as with agitation, shaking, stirring, mixing, horizontal motion, rocking, and others.

[00178] In some embodiments of the present invention, a matrix may be lyophilized before at least one modified growth factor or compositions described herein are administered to it. In certain embodiments, one or more modified growth factors may be administered to a matrix, and the treated matrix may be subsequently lyophilized. The lyophilized, treated matrix can then be rehydrated before it is used. Further, the cells can be seeded onto the matrix before implantation.

[00179] Examples of suitable osteoconductive, chondroconductive matrices include but are not limited to, collagen (*e.g.*, bovine dermal collagen), fibrin, calcium phosphate ceramics (*e.g.*, hydroxyapatite and tricalcium phosphate), calcium sulfate, guanidine-extracted allogenic bone and combinations thereof. A number of suitable matrices are commercially available, such as Collograft™ (Collagen Corporation), which is a mixture of hydroxyapatite, tricalcium phosphate and fibrillar collagen, and Interpore™ (Interpore International), which is a hydroxyapatite biomatrix formed by the conversion of marine coral calcium carbonate to crystalline hydroxyapatite.

[00180] A number of synthetic biodegradable polymers can serve as osteoconductive or chondroconductive matrices with sustained release characteristics. Descriptions of these polymers can be found in Behravesh (1999) Clinical Orthopaedics 367, S118 and Lu (2000) Polymeric Delivery Vehicles for Bone Growth Factors in Controlled Drug Delivery: Designing Technologies for the Future, Park and Mrsny eds., American Chemical Society. Examples of these polymers include poly α -hydroxy esters such as polylactic acid/polyglycolic acid homopolymers and copolymers, polyphosphazenes (PPHOS), polyanhydrides and poly(propylene fumarates).

[00181] Polylactic acid/polyglycolic acid (PLGA) homo and copolymers are well known in the art as sustained release vehicles. The rate of release can be adjusted by the skilled artisan by variation of polylactic acid to polyglycolic acid ratio and the molecular weight of the polymer (see Anderson

(1997) Adv. Drug Deliv. Rev. 28:5. The incorporation of PEG into the polymer as a blend to form microparticle matrices allows further alteration of the release profile of the active ingredient (see Cleek (1997) J. Control Release 48, 259). Ceramics such as calcium phosphate and hydroxyapatite can also be incorporated into the formulation to improve mechanical qualities.

[00182] In one embodiment, the matrices used in the compositions and methods of the present invention are bone matrices. As used herein, a bone matrix is a matrix derived from or including elements of natural bone. In some embodiments, the natural bone is mineralized, partially demineralized, demineralized, cancellous, cortical, or cortical cancellous bone. The bone matrices used herein may or may not include additional synthetic components not typically found in bone tissue. Other embodiments include compositions and methods utilizing a matrix derived from cartilage, other soft tissues such as the dermis, connective tissue, fascia, small intestine submucosa, serous membrane, pericardium, tendon, ligament, muscle, adipose tissue, myelin, blood vessels, base membrane, amniotic membrane and others. A matrix prepared from hyaline cartilage, fibrocartilage or elastic cartilage, may be employed in some embodiments. A matrix may be prepared from hyaline cartilage found in the condyle, tibial plateau, femoral head, humeral head, costal cartilage, or fibrocartilage found in intervertebral discs, or elastic cartilage found in the epiglottis or ear. In certain embodiments, a matrix derived from natural sources that has been optionally cleaned, disinfected, chemically modified, decellularized, particulated, homogenized, lyophilized, gamma irradiated, and/or plasticized may be used. Any of the matrices used herein may or may not include additional synthetic components not typically found in such tissue.

[00183] In one specific embodiment, the bone matrices or cartilage matrices may be demineralized or decellularized, respectively. Examples of demineralized matrices and methods of making are described in U.S. Patent No. 6,189,537 and 6,305,379.

[00184] The matrix, tissue, or organ used in certain embodiments of the present invention may be in the form of a powder, particulates, sheets, fibers, gels, putties, paste, blocks, cylinders, sponges, meshes, films, slices, curls, flakes, or wedges, among others. In certain embodiments of the present invention the matrix, tissue, or organ comprising the modified growth factor peptide(s) may be in the form of a powder, fibers, putty, or a sponge. In further embodiments, the sponge can include, for example, the implant having sponge-like structures disclosed in the co-pending, commonly-assigned patent application PCT/US09/04556 entitled "Composition for a Tissue Repair Implant and Methods of Making the Same" filed on August 7, 2009. The treated matrices can be used in any of the methods of the present invention.

[00185] The invention also relates to methods of increasing or promoting osteogenesis, chondrogenesis, or ligament/tendon genesis in cells. The methods may comprise treating the cells in matrices with at least one of the modified growth factor peptides, or compositions described herein.

As used herein, “osteogenesis” is the deposition new bone material or formation of new bone, including, but not limited to, intramembranous osteogenesis and endochondral osteogenesis. As used herein, “chondrogenesis” is the deposition new cartilage material or formation of new cartilage. As used herein, “ligament/tendon genesis” is the deposition new ligament and/or tendon material or formation of new ligament and/or tendon. The osteogenic, chondrogenic, ligament, or tendon inducing activity of the novel peptides may or may not be altered, including but not limited to, enhanced activity, relative to the normal, mature growth factor. The cells to which the modified growth factors may be administered include cells in any tissue in which bone, cartilage, ligament, or tendon formation is desired, such as, but not limited to, bone, cartilage, ligament, muscle, tendon, etc.

[00186] The invention also relates to methods of treating a tissue or organ defect or injury, for example, a musculoskeletal, dental or soft-tissue defect or injury, in an animal comprising administering (1) cells cultured in the presence of one or more modified growth factors, or compositions described herein and/or (2) one or more modified growth factors, or compositions described herein to the tissue or organ defect (*e.g.* osseous defects, defects in cartilage, ligament, tendon, spine disk, and tendon insertion site to bone).

[00187] The invention further relates to methods of treating a tissue or an organ defect or injury, for example a musculoskeletal, dental or soft-tissue defect, in an animal by applying a treated matrix to the defect, and application to the defect may be accomplished by injecting the treated matrix into the defect, inserting the treated matrix between tissue or organ, or placing the treated matrix on top of the defect. The present invention is also directed to treating a defect or injury in an organ in a similar manner. At least one cofactor may be added to a treated matrix, such as a matrix comprising a modified growth factor, in some embodiments.

[00188] In yet another embodiment, cells may be seeded onto a treated matrix. The cells seeded on the treated matrix can be any cell, such as but not limited to, osteoblasts, chondrocytes, ligament cells, tendon cells, progenitor cells, and stem cells disclosed herein or otherwise known in the art. The seeded cells may be allowed to proliferate and possibly attach to the matrix. Methods of seeding cells onto matrices, such as collagen, are well known in the art. Alternatively, cells may first be treated with at least one modified growth factor or compositions described herein and the treated cells may then be seeded onto a treated or untreated matrix.

[00189] Any of the methods of the present invention can be performed in virtually any setting, such as an in vivo, ex vivo, in situ or in vitro setting. For example, methods of promoting osteogenesis, chondrogenesis, or tendon/ligament inducing activities in cells may be performed in cell culture, may be performed in seeded cells on matrix, or may be performed in an intact organism. Moreover, any combination of any two or more of any of the embodiments described herein are contemplated.

[00190] While the invention has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the invention is not restricted to the particular combinations of material and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the invention being indicated by the following claims. All references, patents and patent applications referred to in this application are herein incorporated by reference in their entirety.

[00191] The following examples are illustrative and are not intended to limit the scope of the invention described herein.

[00192] Example 1 – Preparation and Sequencing of Modified BMP-2

[00193] Collagenase (Sigma Aldrich) was linked to sepharose beads and the un-bound collagenase was removed by extensive washing. Collagenase linked-sepharose beads were used to treat recombinant human BMP-2 (rhBMP-2) with incubation at 37°C overnight with gentle mixing. The rhBMP-2 had the amino acid sequence of amino acids 283-396 of SEQ ID NO: 1 prior to treatment. The modified mature rhBMP-2 and unmodified mature rhBMP-2 control were separated by SDS-PAGE gel electrophoresis and stained by silver staining. The related bands were cut and sequenced using MALDI-TOF mass spectrometry.

[00194] The results of MALDI-TOF mass spectrometry of the collagenase-treated rhBMP-2 sequencing revealed the same 13 amino acids on the C-terminus as the last 13 amino acids of carboxyl-terminus of untreated mature rhBMP-2. The MALDI-TOF sequencing confirms that the collagenase treatment did not truncate the C-terminus of the rhBMP-2.

[00195] Example 2 – Preparation and Sequencing of Modified BMP-2

[00196] The mature rhBMP-2 was treated with trypsin or collagenase in a microcentrifuge tube at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 °C. The treated mature rhBMP-2 was further purified by reverse phase HPLC using a C-18 column. The collected fractions from HPLC were tested for osteoinductive potential using an in vitro alkaline phosphatase assay. The fractions that showed significantly high alkaline phosphatase activities were further separated by SDS-PAGE gel electrophoresis and blotted onto PVDF membrane. Proteins on the PVDF membrane were stained by coomassie blue and the related bands were cut and sent for N-terminal sequencing..

[00197] In the Coomassie blue stained PVDF membrane, the fraction B43-44 sample from the trypsin-treated rhBMP-2 and the fraction A42-44 sample from the collagenase-treated rhBMP-2 showed clean bands at around 14-15 kDa.

[00198] Nine out of ten amino acids of the N-terminus of the trypsin-treated mature rhBMP-2 and eight out of the ten amino acids of the N-terminus of the collagenase-treated mature rhBMP-2 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the trypsin-treated rhBMP-2 was SSCKRHPLYV, which is the first 10 amino acids of SEQ ID NO:16 herein, and the deduced amino acid sequence of the N-terminus of the collagenase-treated rhBMP-2 was LKSSCKRHPL, which is the first 10 amino acids of SEQ ID NO:18 herein. The N-terminus of the trypsin-treated rhBMP-2 matches the untreated mature rhBMP-2 sequence after the 11th amino acid of the N-terminus, indicating that the trypsin cleaved the mature BMP-2 after the first 11 amino acids. Similarly, the N-terminus of the collagenase-treated rhBMP-2 matches the untreated mature rhBMP-2 sequence after the 9th amino acid of the N-terminus, indicating that the collagenase cleaved the mature BMP-2 after the first 9 amino acids. Western blot detected untreated and trypsin or collagenase-treated mature rhBMP-2, and the treated rhBMP-2 was about 1-2 kDa smaller than the untreated rhBMP-2.

[00199] Example 3 – Osteoinductive Potential of Modified rhBMP-2

[00200] Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37 °C. The alkaline phosphatase activities of the HPLC fractions from Example 2 are shown in Figures 1 and 2. The fraction B43-44 from the trypsin-treated rhBMP-2 and the fraction A42-44 from the collagenase treated rhBMP-2 showed significantly higher alkaline phosphatase activity compared to other HPLC fractions. These results along with Figure 3 described below demonstrate that trypsin or collagenase-treated mature rhBMP-2, having an N-terminal truncation, facilitates an increase in alkaline phosphatase activity in vitro.

[00201] Example 4 – Osteoinductive Potential of Modified rhBMP-2 with or without a Protease

[00202] C2C12 cells (ATCC CRL-1772), which are from a mouse myoblast cell line, were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. Collagenase and rhBMP-2 were mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 °C. The treated mature rhBMP-2 was aliquoted with or without further HPLC purification described above. The aliquot with further HPLC purification (purified modified BMP2) was quantified by ELISA. The aliquot without further HPLC purification (non-purified modified

BMP2) retained the collagenase in the aliquot. The aliquots were used to test the effect of treated mature rhBMP-2 on osteogenic potential of C2C12 myoblasts.

[00203] Each of the rhBMP-2 control, and the purified modified rhBMP-2, and the non-purified modified rhBMP-2 at the same concentration was introduced into wells of the C2C12 cell seeded 24-well plate. C2C12 cells cultured in media alone (without addition of rhBMP-2 or modified rhBMP-2) were used as a negative control.

[00204] After 3 days incubation at 37 °C, 5% CO₂, the AP assay as described above was performed, and the results are shown in Figure 3. The alkaline phosphatase activity of cells cultured with the purified modified rhBMP-2 or the non-purified modified rhBMP-2 were about 39 times and 103 times higher than that of cells cultured with non-treated rhBMP-2 control, respectively. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-2 was about 1.6 times higher than that of cells cultured with purified modified rhBMP-2. Thus, the results demonstrate a significant increase in the alkaline phosphatase activity by administering the non-purified modified rhBMP-2 compared to administering the purified modified rhBMP-2.

[00205] Further, with another set of C2C12 cells cultured as described above, the purified modified rhBMP-2 was introduced into wells of the C2C12 cell along with 138 ng/mL of active dispase or inactive dispase, or 100 ug/mL collagenase or inactive collagenase. The AP assay as described above was performed, and the results are demonstrated in Figure 4. The results showed that adding active or inactive dispase or collagenase to the purified modified rhBMP-2 samples also increased the alkaline phosphatase activity.

[00206] In addition, rhBMP-2 treated by clostripain, dispase, and the mixture thereof, without further purification, also increased the alkaline phosphatase activity compared to non-modified rhBMP-2 control. In addition, rhBMP-2 treated by BMP1 or MMP13, without further purification, also increased the alkaline phosphatase activity compared to non-modified rhBMP-2 control.

[00207] Example 5 – Osteoinductive Potential of Modified rhBMP-2 with a Protease

[00208] C2C12 cells (ATCC CRL-1772) were cultured as described above. Collagenase and rhBMP-2 were mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 °C with agitation. The treated mature rhBMP-2 was aliquoted, and the aliquots were used to test the effect of treated mature rhBMP-2 on osteogenic potential of C2C12 myoblasts as follows.

[00209] For the osteoinductive potential assessment, base media of DMEM containing 1% FBS, 50 µg/mL of ascorbic acid and 10 mM of β-glycerolphosphate was used as a control group. The base

media control with the addition of 50 ng/mL of rhBMP-2 was used as the BMP-2 positive control group. The base media control with the addition of 50 ng/mL of modified rhBMP-2 without the purification was used as the test group. The media were added into C2C12 cells seeded on chamber slides and changed every 3-4 days.

[00210] After 4 days incubation at 37 °C, 5% CO₂, photographs were also taken for cell cultures in each group. The nodule formation of C2C12 cells appeared only in modified rhBMP-2 group, but not in the other two control groups. After 6 or 12 days of incubation, the media was removed from chamber slides and the slides were stained with Alizarin Red S. Alizarin red S has been used to identify calcium-rich deposits by cells in culture or tissue sections, which indicates the effect of osteoinductive material on osteogenic potential of cells.

[00211] For the base media control group, no positive Alizarin red S staining was found at any tested time points. For the rhBMP-2 control group, some Alizarin red S staining was detected after 6 or 12 days of incubation. For the modified rhBMP-2 group, after 6 days of incubation, significant amount of nodules stained positive by Alizarin red S. After 12 days of incubation, more Alizarin red S positively stained nodules of larger size were observed in C2C12 cells cultured with modified rhBMP-2 than that in C2C12 cells cultured with unmodified rhBMP-2. This data suggests that modified rhBMP-2 has a significantly greater effect on osteogenic potential of C2C12 myoblasts as compared to the unmodified mature rhBMP-2 control.

[00212] Example 6 – Alkaline Phosphatase Assay for Cells Cultured with Modified rhBMP-2

[00213] Alkaline phosphatase is one of the distinctive indicators of osteoinductivity. C2C12 cells (ATCC CRL-1772), which are from a mouse myoblast cell line, were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. Collagenase and rhBMP-2 were mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37°C with agitation. On day two, the collagenase-treated rhBMP-2, untreated rhBMP-2 at the same concentration as treated rhBMP-2, or collagenase alone at the same concentration of collagenase used to treat rhBMP-2 was introduced into each well of the C2C12 cell seeded 24-well plate. C2C12 cells cultured without the addition of rhBMP-2 or collagenase were used as a negative control. After 3 days of incubation at 37°C, 5% CO₂, cells were collected from the culture plates, and cell lysates were prepared for AP assays and bicinchoninic acid (BCA) total protein assays (BCA reagent available from Pierce Biotechnology). The AP assay was performed as described above. The BCA assay is a detergent-compatible protein assay used for colorimetric detection and quantitation of total protein. The results of the in vitro AP assay are shown in Table IV. The alkaline phosphatase activity of cells cultured with collagenase-treated rhBMP-2 was about 87 times higher than that of cells cultured with untreated rhBMP-2.

[00214] Table 6. Alkaline phosphatase activity of cells

Groups	Negative Control	Unmodified rhBMP-2	Collagenase Control	Modified rhBMP-2
Mean AP activity (nmol pNP/min/mg protein)	0.86 ± 0.08	9.93 ± 0.08	1.02 ± 0.29	862.31 ± 40.71

[00215] Example 7 – Osteoinductive potential of mature rhBMP-2 with the addition of protease

[00216] C2C12 mouse myoblast cell line (ATCC CRL-1772), were cultured as described above. The mature rhBMP-2 (50 ng/mL, R&D Systems, Inc.), or the same concentration of mature rhBMP-2 with the addition of dispase or collagenase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of rhBMP-2) were used as a negative control.

[00217] After 3 days incubation at 37 °C, 5% CO₂, an AP assay was performed as described above. The alkaline phosphatase activities in cells induced by the mature rhBMP-2 in combination with either dispase or collagenase were about 24% and 293% higher than that induced by the mature rhBMP-2 without addition of protease, respectively.

[00218] Example 8 – Preparation and Sequencing of Modified BMP-4

[00219] Collagenase (Sigma Aldrich) was linked to sepharose beads and the un-bound collagenase was removed by extensive washing. Collagenase linked-sepharose beads were used to treat mature rhBMP-4 by incubation at 37 °C overnight with gentle mixing. The treated mature rhBMP-4 was separated by SDS-PAGE gel electrophoresis followed by protein transferring onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue and the related bands were cut for N-terminal sequencing using Edman degradation chemistry with an ABI Procise 494 sequencer. Proteins on PVDF membrane were also detected by Western Blot using antibody against C-terminus of rhBMP-4.

[00220] By comparing the collagenase treated mature rhBMP-4 bands with the untreated mature rhBMP-4 control bands, extra bands at around 17-22 kDa were cut for N-terminal sequencing. Ten amino acids of the N-terminus of the collagenase treated mature rhBMP-4 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the treated rhBMP-4 was KKNKNCRRHS, which is the first 10 amino acids of SEQ ID NO:19 herein. The N-terminus of the treated rhBMP-4 matched the untreated mature rhBMP-4 sequence, indicating that the collagenase cleaved the mature BMP-4 after the first 10 amino

acids. The Western blot detected a band for collagenase treated rhBMP-4 group, which is about 1-2 kDa smaller than the band for untreated mature rhBMP-4.

[00221] Example 9 – Osteoinductive Potential of Modified rhBMP-4

[00222] Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37 °C. C2C12 cells (ATCC CRL-1772) were seeded at a density of about 25,000 cells/cm² in 24-well plates on day one. Collagenase and various concentrations of mature rhBMP4 were mixed in microcentrifuge tubes at molarity ratios between about 1:10 and about 1000:1 and incubated at 37 °C overnight. The collagenase-treated rhBMP-4 or untreated rhBMP4 was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP4 were 1, 5, 10, and 30 ng/mL. After 3 days of incubation at 37 °C and 5% CO₂, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays.

[00223] The results of the in vitro AP assay are shown in Figure 5. When cells were cultured with treated or untreated mature rhBMP-4 at a concentration of 10 ng/mL or less, the differences in alkaline phosphatase activity were negligible. At a concentration of 30 ng/mL, cells treated with collagenase-treated rhBMP-4 expressed significantly higher AP activities than those treated with untreated mature rhBMP-4.

[00224] Example 10 – Osteoinductive Potential of Modified rhBMP-4 with or without a Protease

[00225] C2C12 cells (ATCC CRL-1772), which are from a mouse myoblast cell line, are seeded at a density of 25,000 cells/cm² in 24-well plates on day one. Collagenase and rhBMP-4 are mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and are incubated overnight at 37 °C. The treated mature rhBMP-4 is aliquoted with or without further HPLC purification described above. The aliquot with further HPLC purification (purified modified BMP-4) is quantified by ELISA. The aliquot without further HPLC purification (non-purified modified BMP-4) retained the collagenase in the aliquot. The aliquots are used to test the effect of treated mature rhBMP-4 on osteogenic potential of C2C12 myoblasts.

[00226] Each of the rhBMP-4 control, and the purified modified rhBMP-4, and the non-purified modified rhBMP-4 at the same concentration is introduced into wells of the C2C12 cell seeded 24-well plate. C2C12 cells cultured in media alone (without addition of rhBMP-4 or modified rhBMP-4) are used as a negative control.

[00227] After 3 days incubation at 37 °C, 5% CO₂, the AP assay as described above is performed. The alkaline phosphatase activity of cells cultured with the purified modified rhBMP-4 or the non-purified modified rhBMP-4 are significantly higher than that of cells cultured with non-treated rhBMP-4

control. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-4 is also significantly higher than that of cells cultured with purified modified rhBMP-4. The results demonstrate a significant increase in the alkaline phosphatase activity by administering the non-purified modified rhBMP-4 compared to administering the purified modified rhBMP-4.

[00228] Example 11 – Preparation and Sequencing of Modified BMP-5

[00229] Modified recombinant human BMP-5 (rhBMP-5) was obtained by treating mature rhBMP-5 with dispase (Worthington), collagenase (Sigma-Aldrich), or cathepsin K. The mature rhBMP-5 had the sequence of amino acids 317-454 of SEQ ID NO:4 prior to modification. Aliquots of the modified mature rhBMP-5 and mature rhBMP-5 control were separated by SDS-PAGE gel electrophoresis and the peptides were transferred onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue or detected with Western blot using antibody against amino acids 407-450 of rhBMP-5.

[00230] The related bands from Coomassie blue stained PVDF membrane were cut and used for N-terminal sequencing. Ten amino acids of the N-terminus of the dispase-modified or collagenase-modified mature rhBMP-5 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the modified rhBMP-5 was taken to be YNTSEQKQAC, which is the first 10 amino acids of SEQ ID NO:21 herein. Another deduced amino acid sequence of the N-terminus of the modified rhBMP-5 was taken to be SVGDYNTSEQ, which is the first 10 amino acids of SEQ ID NO:20 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be TSEQKQACKK, which is the first 10 amino acids of SEQ ID NO:22 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be DYNTSEQKQA, which is the first 10 amino acids of SEQ ID NO:23 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be MSSVGDYNTS, which is the first 10 amino acids of SEQ ID NO:24 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be VGDYNTSEQK, which is the first 10 amino acids of SEQ ID NO:25 herein. Another deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be QA(C)KKHELYV, which is the first 10 amino acids of SEQ ID NO:26 herein. One more deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be EQKQA(C)KKHE, which is the first 10 amino acids of SEQ ID NO:27 herein. One more deduced amino acid sequence of the N-terminus of the treated rhBMP-5 was taken to be SEQKQACKKH, which is the first 10 amino acids of SEQ ID NO:28 herein.

[00231] Example 12 – Osteoinductive Potential of Modified rhBMP-5

[00232] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. Dispase or collagenase and rhBMP-5 were mixed in microcentrifuge tubes at molarity ratios between about 1:100 and about 1000 :1 and incubated at 37°C for 10-60 minutes or 18-20 hours. The modified rhBMP5 or unmodified rhBMP5 was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP5 were 0.25 ug/mL, 0.5 ug/mL, and 1 ug/mL in each well of control and modified groups. After 3 days of incubation at 37°C and 5% CO₂, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays. Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37°C.

[00233] The results of the in vitro AP assay are shown in Figures 6 and 7. When cells were cultured with dispase-modified or unmodified rhBMP5 at a concentration of 0.25 ug/mL, 0.5 ug/mL, or 1 ug/mL, the alkaline phosphatase activity induced by dispase-modified rhBMP5 was about 400 times, 120 times, or 35 times higher than that induced by unmodified rhBMP5, respectively (Fig. 6). When cells were cultured with collagenase-modified or unmodified rhBMP5, significant increases on alkaline phosphatase activity were found in all concentration groups (Fig. 7). At 0.25 ug/mL, 0.5 ug/mL, or 1 ug/mL in cell culture, the alkaline phosphatase activity induced by collagenase-modified rhBMP5 was about 1000 times, 300 times, or 50 times higher than that induced by unmodified rhBMP5, respectively.

[00234] Example 13 – Osteoinductive Potential of Modified rhBMP-5 with or without a Protease

[00235] Dispase and rhBMP-5 were mixed in microcentrifuge tubes at a molarity ratio of between about 1:1000 and about 100:1 and incubated at 20 to 37 °C for 10-60 minutes. The treated mature rhBMP-5 was further purified with reverse phase HPLC using a C-18 column. The collected fractions from HPLC were lyophilized, quantified and used to test the effect of modified rhBMP-5 on osteoinductive potential of C2C12 myoblasts.

[00236] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. The purified modified rhBMP-5 alone, or the same concentration of purified modified rhBMP-5 with the addition of dispase or collagenase was introduced into wells of C2C12 cell. C2C12 cells cultured in media alone (without addition of modified rhBMP-5) were used as a negative control.

[00237] After 3 days incubation at 37 °C, 5% CO₂, an AP assay was performed as described above. As shown in Figure 8, Alkaline phosphatase activity in the cells induced by the purified modified

rhBMP-5 in combination with dispase was about 76% higher than that induced by the purified modified rhBMP-5 alone. Alkaline phosphatase activity in the cells induced by the purified modified rhBMP-5 in combination with collagenase was about 176% higher than that induced by the purified modified rhBMP-5 alone.

[00238] Example 14 – Chondrogenic Potential of Modified rhBMP-5

[00239] The ATDC5 murine chondrosarcoma cell line (Riken Cell Bank) that have been widely used to study chondrocyte differentiation were seeded at 2,000 cells/cm² in 6-well plates. After 5 days of culture, the culture media was changed to the differentiation media that contains 5% fetal bovine serum, 10 ug/mL bovine insulin, and 50 ug/mL ascorbic acid (differentiation media control), or the differentiation media with the addition of rhBMP-5 or dispase-modified rhBMP-5 prepared as shown above. The cells were cultured in the differentiation media for a total of 13 days and the media was changed every 3 days. Cells were collected at different time points and total RNA was extracted and followed by reverse transcription using Applied Biosystems RNA-to-cDNA kit. The chondrogenic-specific genes, collagen type 2a (COL2a1), aggrecan (ACAN), cartilage oligomeric matrix protein (COMP) and transcription factor SOX9, were quantified using TaqMan Gene Expression Assay kit from Applied Biosystems, Inc. The relative expression (ΔC_t) of the target gene was normalized to GAPDH endogenous gene expression and the fold change ($2^{-\Delta\Delta C_t}$) was calculated relative to the differentiation media control. As shown in tables 7-10 below, the COL2a1, ACAN, COMP, SOX9 gene expressions in cells cultured with dispase-modified rhBMP-5 were all higher than that in cells cultured with rhBMP-5 control.

Table 7. Expression of COL2a1

Incubation Time (days)	rhBMP-5 Control	Modified rhBMP-5	
	Fold change relative to differentiated media control	Fold change relative to differentiated media control	% of increase
3	1.30 ±0.21	2.08 ±0.19	60.58
5	1.23 ±0.09	1.07 ±0.08	-12.94
7	0.70 ±0.06	1.85 ±0.57	163.29
9	0.84 ±0.05	1.17 ±0.10	39.80
11	1.52 ±0.19	3.40 ±0.43	123.97
13	1.38 ±0.05	3.38 ±0.19	145.09

[00240] Table 8. Expression of ACAN

Incubation Time (days)	rhBMP-5 Control	Modified rhBMP-5	
	Fold change relative to differentiated media control	Fold change relative to differentiated media control	% of increase
3	0.85 ±0.08	1.94 ±0.10	127.10
5	0.78 ±0.05	2.71 ±0.22	249.03

7	0.83 ±0.04	3.46 ±1.06	316.02
9	0.35 ±0.01	3.90 ±0.16	1000.43
11	1.97 ±0.22	4.50 ±0.56	128.15
13	4.16 ±0.18	13.15 ±0.82	216.02

[00241] Table 9. Expression of COMP

Incubation Time (days)	rhBMP-5 Control	Modified rhBMP-5	% of increase
	Fold change relative to differentiated media control	Fold change relative to differentiated media control	
3	0.55 ±0.06	1.27 ±0.06	130.80
5	0.36 ±0.03	0.23 ±0.03	-37.58
7	0.37 ±0.03	0.92 ±0.28	146.80
9	0.16 ±0.08	0.89 ±0.18	459.19
11	0.47 ±0.06	1.46 ±0.17	210.23
13	0.91 ±0.06	1.77 ±0.17	94.08

Table 10. Expression of SOX9

Incubation Time (days)	rhBMP-5 Control	Modified rhBMP-5	% of increase
	Fold change relative to differentiated media control	Fold change relative to differentiated media control	
3	0.60 ±0.05	0.67 ±0.07	10.70
5	0.59 ±0.18	0.89 ±0.06	50.18
7	0.80 ±0.05	0.71 ±0.21	-11.32
9	0.61 ±0.04	0.83 ±0.07	35.97
11	1.08 ±0.13	1.18 ±0.16	8.92
13	1.44 ±0.26	2.84 ±0.16	97.25

[00242] Example 15-Osteoinductive potential of mature rhBMP-5 with the addition of protease

[00243] C2C12 cells (ATCC CRL-1772) were cultured as described above. The mature rhBMP-5 control (200 ng/mL, R&D Systems, Inc.), or the same concentration of mature rhBMP-5 with the addition of collagenase or dispase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of rhBMP-5) were used as a negative control.

[00244] After 3 days incubation at 37 °C, 5% CO₂, an AP assay was performed as described above. The results showed that the alkaline phosphatase activities in cells induced by the mature rhBMP-5 in

combination with either collagenase or dispase were 21 and 42 times higher than that induced by the mature rhBMP-5 without addition of protease, respectively.

[00245] Example 16 – Preparation and Sequencing of Collagenase-Modified BMP-7

[00246] The mature rhBMP-7 was treated with collagenase in a microcentrifuge tube at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 °C. The treated mature rhBMP-7 was further purified by reverse phase HPLC using a C-18 column. The collected fractions from HPLC were tested for osteoinductive potential using an in vitro alkaline phosphatase assay. The fractions that showed significantly high alkaline phosphatase activities were further separated by SDS-PAGE gel electrophoresis and blotted onto PVDF membrane. Proteins on the PVDF membrane were stained by coomassie blue and the related bands were cut and sent for N-terminal sequencing.

[00247] In the Coomassie blue stained PVDF membrane, the fraction 43 sample from the collagenase-treated rhBMP-7 showed clean bands at around 15-16 kDa.

[00248] Ten amino acids of the N-terminus of the collagenase treated mature rhBMP-7 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the treated rhBMP-7 was taken to be ENSSSDQRQA, which is the first 10 amino acids of SEQ ID NO:31 herein. This deduced N-terminus of the treated rhBMP-7 matches the untreated mature rhBMP-7 sequence except, indicating that the collagenase cleaved the mature BMP-7 after the first 27 amino acids. Western blot detected untreated and collagenase-treated mature rhBMP-7, and the treated rhBMP-7 was about 2-3 kDa smaller than the untreated rhBMP-7.

[00249] Example 17 – Osteoinductive Potential of Collagenase-Modified BMP-7

[00250] Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37 °C. The alkaline phosphatase activities induced by the HPLC fractions from Example 16 are shown in Figure 9. The alkaline phosphatase activity induced by the fraction 43 from the collagen-treated rhBMP-7 was significantly higher than the alkaline phosphatase activity induced by other HPLC fractions.

[00251] Example 18 – Dose Responsiveness of Modified rhBMP-7

[00252] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. Collagenase and various concentrations of rhBMP7 were mixed in microcentrifuge tubes at molarity ratios between about 1:10 and about 1000 :1 and incubated at 37°C overnight. The

collagenase-treated rhBMP7 or untreated rhBMP7 was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP7 were 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL in each well. After 3 days of incubation at 37°C and 5% CO₂, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays.

[00253] The results of the in vitro AP assay are shown in Figure 10. When cells were cultured with treated or untreated rhBMP7 at a concentration of 10 ng/mL or less, the differences in alkaline phosphatase activity were negligible. At concentrations of 25 ng/mL or higher, significant differences were found between collagenase-treated rhBMP7 groups and untreated rhBMP7 groups. At 50 ng/mL in cell culture, the alkaline phosphatase activity induced by collagenase-treated rhBMP7 was about 80 times higher than that induced by untreated rhBMP7.

[00254] Example 19 – Preparation and Sequencing of Trypsin-Modified BMP-7

[00255] rhBMP-7 was incubated with trypsin overnight at 37°C. Trypsin and rhBMP-7 were used at a molarity ratio between about 1:50 and about 100:1. The treated mature rhBMP-7 was separated by SDS-PAGE gel electrophoresis followed by protein transferring onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue and the related bands were cut for N-terminal sequencing using Edman degradation chemistry with an ABI Procise 494 sequencer. Proteins on PVDF membrane were also detected by Western Blot using antibody against C-terminus of rhBMP-7.

[00256] By comparing the trypsin treated mature rhBMP-7 bands with the untreated mature rhBMP-7 control bands, extra band at around 14-16 kDa were cut for N-terminal sequencing. Ten amino acids of the N-terminus of the trypsin treated mature rhBMP-7 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the treated rhBMP-7 was QACKKHELYV, which is the first 10 amino acids of SEQ ID NO:30 herein. The N-terminus of the trypsin-treated rhBMP-7 matches the untreated mature rhBMP-7 sequence, indicating that the trypsin cleaved the mature BMP-7 after the first 35 amino acids. The Western blot detected a band for trypsin treated rhBMP-7 group, which is about 2-3 kDa smaller than the band for untreated mature rhBMP-7.

[00257] Example 20 – Osteoinductive Potential of Trypsin-Modified BMP-7

[00258] C2C12 cells were cultured as described above. Trypsin and rhBMP7 were mixed in a microcentrifuge tube at molarity ratios between about 1:50 and about 100 :1 and incubated at 37°C overnight. The trypsin-modified rhBMP7 or unmodified rhBMP7 was introduced into each well of the C2C12 cell seeded 24-well plate on day two. After 3 days of incubation at 37 °C and 5% CO₂,

cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays.

[00259] The results of the in vitro AP assay are shown in Figure 11. The alkaline phosphatase activity induced by trypsin-modified rhBMP7 was about 1.5 times higher than that induced by unmodified rhBMP7.

[00260] Example 21 – Osteoinductive Potential of Modified rhBMP-7 with or without a Protease

[00261] C2C12 cells were cultured as described above. Collagenase and rhBMP-7 are mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and are incubated overnight at 37 °C. The treated mature rhBMP-7 is aliquoted with or without further HPLC purification described above. The aliquot with further HPLC purification (purified modified BMP-7) is quantified by ELISA. The aliquot without further HPLC purification (non-purified modified BMP-7) retained the collagenase in the aliquot. The aliquots are used to test the effect of treated mature rhBMP-7 on osteogenic potential of C2C12 myoblasts.

[00262] Each of the rhBMP-7 control, and the purified modified rhBMP-7, and the non-purified modified rhBMP-7 at the same concentration is introduced into wells of the C2C12 cell seeded 24-well plate. C2C12 cells cultured in media alone (without addition of rhBMP-7 or modified rhBMP-7) are used as a negative control.

[00263] After 3 days incubation at 37 °C, 5% CO₂, the AP assay as described above is performed. The alkaline phosphatase activity of cells cultured with the purified modified rhBMP-7 or the non-purified modified rhBMP-7 are significantly higher than that of cells cultured with non-treated rhBMP-7 control. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-7 is also significantly higher than that of cells cultured with purified modified rhBMP-7. The results demonstrate a significant increase in the alkaline phosphatase activity by administering the non-purified modified rhBMP-7 compared to administering the purified modified rhBMP-7.

[00264] Example 22 – Osteoinductive Potential of Modified rhBMP-7 with or without an additional Protease

[00265] Dispace and rhBMP-7 were mixed in microcentrifuge tubes at a molarity ratio between about 1:100 and about 1000:1 and incubated at 37°C from about 15 minutes to about 24 hours.

[00266] C2C12 cells were cultured as described above. The non-treated rhBMP-7 control, dispace modified rhBMP-7 without further purification, or dispace modified rhBMP-7 with the addition of 100ug/mL collagenase in culture media were introduced into wells of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured in media alone (without addition of rhBMP-7 or treated rhBMP-7) were used as a negative control. After 3 days of incubation at 37°C/5% CO₂, cells were

collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays as described above.

[00267] The results of the in vitro AP assay are shown in Figure 12. The alkaline phosphatase activity of cells cultured with dispase modified rhBMP-7 was about 141% higher than that of cells cultured with non-treated rhBMP-7 control, while the alkaline phosphatase activity of cells cultured with dispase modified rhBMP-7 with the addition of collagenase in media further increased 116% compared to the dispase modified rhBMP-7 group. The alkaline phosphatase activity of cells cultured with dispase modified rhBMP-7 with the addition of collagenase in media was about 421% higher than that of cells cultured with non-treated rhBMP-7 control.

[00268] Example 23 – Osteoinductive Potential of Modified rhBMP-7 with a Protease

[00269] C2C12 cells (ATCC CRL-1772) are cultured as described above. Collagenase and rhBMP-7 are mixed in microcentrifuge tubes at a molarity ratio of between about 1:10 and about 100:1 and incubated overnight at 37 °C with agitation. The treated mature rhBMP-7 is aliquoted, and the aliquots are used to test the effect of treated mature rhBMP-7 on osteogenic potential of C2C12 myoblasts as follows.

[00270] For the osteoinductive potential assessment, base media of DMEM containing 1% FBS, 50 µg/mL of ascorbic acid and 10 mM of β-glycerolphosphate is used as a control group. The base media control with the addition of 50 ng/mL of rhBMP-7 is used as the BMP-7 positive control group. The base media control with the addition of 50 ng/mL of modified rhBMP-7 without the purification is used as the test group. The media are added into C2C12 cells seeded on chamber slides and changed every 3-4 days.

[00271] After several days of incubation at 37 °C, 5% CO₂, photographs are also taken for cell cultures in each group. The nodule formation of C2C12 cells appear only in modified rhBMP-7 group, but not in the other two control groups. After more days of incubation, the media is removed from chamber slides and the slides are stained with Alizarin Red S. Alizarin red S is used to identify calcium-rich deposits by cells in culture or tissue sections, which indicates the effect of osteoinductive material on osteogenic potential of cells.

[00272] For the base media control group, no positive Alizarin red S staining is found at any tested time points. For the rhBMP-7 control group, some Alizarin red S staining is detected after 6 or 12 days of incubation. For the modified rhBMP-7 group, after 6 days of incubation, significant amount of nodules stain positive by Alizarin red S. After 12 days of incubation, more Alizarin red S positively stain nodules of larger size are observed in C2C12 cells cultured with modified rhBMP-7 than that in C2C12 cells cultured with unmodified rhBMP-7. This data suggests that modified

rhBMP-7 has a significantly greater effect on osteogenic potential of C2C12 myoblasts as compared to the unmodified mature rhBMP-7 control.

[00273] Example 24 – Preparation and Sequencing of Modified BMP-13

[00274] Modified recombinant human BMP-13 (rhBMP-13 or rhGDF-6) was obtained by treating mature rhBMP-13 with dispase (Worthington) or collagenase (Sigma-Aldrich). The mature rhBMP-13 had the sequence of amino acids 336-455 of SEQ ID NO:12 prior to modification. Aliquots of the modified mature rhBMP-13 and unmodified mature rhBMP-13 control were separated by SDS-PAGE gel electrophoresis and the peptides were transferred onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue or detected with Western blot using antibody against C-terminal region of mature rhBMP-13.

[00275] The related bands from Coomassie blue stained PVDF membrane were cut and used for N-terminal sequencing. Ten amino acids of the N-terminus of the dispase modified mature rhBMP-13 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the modified mature rhBMP-13 was taken to be GKKSRLRCSK, which is the first 10 amino acids of SEQ ID NO:34 herein.

[00276] Example 25 – Improvement of Osteoinductive Potential of Modified BMP-13

[00277] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. Dispace and mature rhBMP-13 (rhGDF-6) were mixed in microcentrifuge tubes at molarity ratios between about 1:100 and about 1000 :1 and incubated at 37°C for 5-60 minutes. The dispace-modified mature rhGDF-6 or unmodified mature rhGDF-6 control was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP-13 were 1 ug/mL, 2 ug/mL, and 3.67 ug/mL for both control and modified groups. After 3 days of incubation at 37°C and 5% CO₂, cells were collected from culture plates and cell lysates were prepared for Alkaline phosphatase (AP) assays and BCA total protein assays. Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37°C.

[00278] The results of the in vitro AP assay are shown in Figure 13. When cells were cultured with dispace-modified or unmodified rhBMP-13 at a concentration of 1 ug/mL or higher, significant enhancements of AP activity were found in cells treated with dispace-modified mature rhBMP-13 compared to the cells treated with unmodified mature rhBMP-13 (Fig.13). The alkaline phosphatase activity induced by dispace-modified mature rhBMP-13 was about 300 - 350 times higher than that induced by unmodified mature rhBMP-13 control.

[00279] Example 26 – Improvement of Osteoinductive Potential of Modified BMP-13

[00280] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. Collagenase (Sigma) and mature rhBMP-13 were mixed in microcentrifuge tubes at molarity ratios between about 1:100 and about 1000 :1 and incubated at 37°C for 0.5-24 hours. The collagenase-modified mature rhBMP-13 or unmodified mature rhBMP-13 control was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP-13 were 0.5 ug/mL, 1 ug/mL, and 2 ug/mL for both control and modified groups. After 3 days of incubation at 37°C and 5% CO₂, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays.

[00281] The results of the in vitro AP assay are shown in Figure 14. When cells were cultured with collagenase-modified or unmodified rhBMP-13 at a concentration of 0.5 ug/mL or higher, significant enhancements of AP activity were found in cells treated with collagenase-modified mature rhBMP-13 compared to the cells treated with unmodified mature rhGDF-6 (Fig.14). The alkaline phosphatase activity induced by collagenase-modified mature rhBMP-13 was about 0.7 - 20 times higher than that induced by unmodified mature rhBMP-13 control.

[00282] Example 27 – Osteoinductive Potential of purified Modified rhBMP-13 with or without a Protease

[00283] Dispase and rhBMP-13 (PeproTech, Inc.) were mixed in microcentrifuge tubes at a molarity ratio of between about 1:100 and about 100:1 and incubated at 35-40 °C for 10-60 minutes. The mixture was purified with reverse phase HPLC using a C-18 column. The HPLC purified modified rhBMP-13 was used to test the effect of modified rhBMP-13 on osteogenic potential of C2C12 myoblasts.

[00284] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. The purified modified rhBMP-13, or the same concentration of purified modified rhBMP-13 with the addition of dispase or collagenase (molarity ratio between about 1:100 and about 100:1), were introduced into wells of the C2C12 cells on day two. C2C12 cells cultured in media alone (without addition of modified rhBMP-13) were used as a negative control.

[00285] After 3 days of incubation at 37 °C, 5% CO₂, the AP assay was performed as described above. The alkaline phosphatase activity in the cells induced by the purified modified rhBMP-13 in combination with either dispase or collagenase was about of 25 times and 50% higher than that induced by purified modified rhBMP-13 alone, respectively.

[00286] Example 28 – Osteoinductive Potential of Modified rhBMP-13 with or without an additional Protease

[00287] C2C12 cells were cultured as described above. Non-purified modified rhBMP-13 (1 ug/mL), or an equal concentration of non-purified modified rhBMP-13 with the addition of either dispase or collagenase was introduced into wells of the C2C12 cells. The AP assay was performed as described above. The alkaline phosphatase activity in the cells induced by the non-purified modified rhBMP-13 in combination with either dispase or collagenase were about 18% and 81% higher than that induced by the non-purified modified rhBMP-13 alone, respectively.

[00288] Example 29 – Osteoinductive potential of mature rhBMP-13 with the addition of protease

C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. The mature rhBMP-13 (1 µg/mL, PeproTech, Inc.), or the same concentration of mature rhBMP-13 with the addition of dispase or collagenase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of rhBMP-13) were used as a negative control.

After 3 days incubation at 37 °C, 5% CO₂, an AP assay was performed as described above. The results showed that the alkaline phosphatase activities in cells induced by the mature rhBMP-13 in combination with either dispase or collagenase were about 50-180 times and 235% higher than that induced by the mature rhBMP-13 without addition of protease, respectively.

[00289] Example 30 – Effect of Modified rhBMP-13 on tendon-specific gene expression of human adipose-derived stem cells

[00290] Human adipose-derived stem cells (hASC) were isolated from fat tissue of a human donor with research consent. Cells were propagated until the 5th passage and seeded at 5,000 cells/cm² in 6-well plates. After one day, the culture media was changed to the differentiation media that contains rhBMP-13 control (100ng/mL) or dispase-modified rhBMP-13 (100ng/mL) prepared as shown above. The cells were cultured in differentiation media for a total of 12 days and the media was changed every 3 days. Cells were collected at different time points and total RNA was extracted and followed by reverse transcription using Applied Biosystems RNA-to-cDNA kit. The target tendon-specific genes, scleraxis (SCX) and tenomodulin (TMND), were amplified and quantified using TaqMan Gene Expression Assay kit from Applied Biosystems, Inc. The relative expression (ΔC_t) of the target gene was normalized to GAPDH endogenous gene expression and the fold change ($2^{-\Delta\Delta C_t}$) was calculated relative to the hASC control.

[00291] As shown in table IV below, the SCX gene expression in cells cultured with dispase-modified rhBMP-13 was about 18% to 40% higher than that in cells cultured with rhBMP-13 control for the first 6 days of culture. On day 9 and day 12, the SCX expression in cells cultured with dispase-modified rhBMP-13 was about 63% and 97% higher than that in cells cultured with rhBMP-13 control. The TMND gene expression in cells cultured with dispase-modified rhBMP-13 was about 84% higher than that in cells cultured with the rhBMP-13 control on day 6.

[00292] Table 11. Expression of Scleraxis (SCX)

Incubation Time (days)	rhBMP-13 Control	Modified rhBMP-13	% of increase
	Fold change relative to differentiated media control	Fold change relative to differentiated media control	
1	1.34 ±0.42	1.62 ±0.71	20.86
2	1.16 ±0.16	1.67 ±0.38	44.73
3	2.05 ±0.67	2.49 ±0.93	21.42
6	1.16 ±0.27	1.37 ±0.13	18.1
9	0.98 ±0.15	1.60 ±0.19	63.58
12	1.52 ±0.24	2.99 ±0.41	97.25

[00293] Example 31 – Preparation and Sequencing of Modified BMP-14

[00294] Modified recombinant human BMP-14 (rhGDF-5 or rhBMP-14) was obtained by treating mature rhBMP-14 with dispase (Worthington) or collagenase (Sigma-Aldrich). The rhBMP-14 had the sequence of amino acids 382-501 of SEQ ID NO:13 prior to modification. Aliquots of the modified mature rhBMP-14 and unmodified mature rhBMP-14 control were separated by SDS-PAGE gel electrophoresis and the peptides were transferred onto PVDF membrane. The proteins on PVDF membrane were stained by Coomassie blue or detected with Western blot using antibody against internal region of mature rhBMP-14.

[00295] The related bands from Coomassie blue stained PVDF membrane were cut and used for N-terminal sequencing. Ten amino acids of the N-terminus of the dispase modified mature rhBMP-14 were detected using Edman degradation chemistry with an ABI Procise® 494 sequencer. The deduced amino acid sequence of the N-terminus of the modified rhBMP-14 was taken to be LKARCSRKAL, which is the first 10 amino acids of SEQ ID NO:35 herein.

[00296] Example 32 – Dose-dependent Change of Osteoinductive Potential of Modified BMP-14

[00297] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. Dispace and rhBMP-14 were mixed in microcentrifuge tubes at molarity ratios between about 1:100 and about 1000 :1 and incubated at 37°C for 5-60 minutes. The dispace-modified rhBMP-14 or unmodified rhBMP-14 control was introduced into each well of the C2C12 cell seeded 24-well plate on day two. The final concentrations of rhBMP-14 were 0.2 ug/mL, 0.5 ug/mL, and 1 ug/mL in each well for both control and modified groups. After 3 days of incubation at 37°C and 5% CO₂, cells were collected from culture plates and cell lysates were prepared for AP assays and BCA total protein assays. Alkaline phosphatase is one of the distinctive biological or biologically-derived indicators of osteoinductivity. The AP assay measures the product para-

nitrophenol (pNP) at a wavelength of 405 nm after 60 minutes of incubation of the substrate para-nitrophenyl phosphate (pNPP) with a cell lysate at 37°C.

[00298] The results of the in vitro AP assay are shown in Figure 15. When cells were cultured with dispase-modified or unmodified rhBMP-14 at a concentration of 0.2 ug/mL or higher, significant enhancements were found in dispase-modified rhBMP-14 groups compared to the unmodified rhBMP-14 control groups (Fig.15). At 0.2 ug/mL, 0.5 ug/mL, or 1 ug/mL in cell culture, the alkaline phosphatase activity induced by dispase-modified rhBMP-14 was about 1.5 times, 9 times, or 19 times higher than that induced by unmodified rhBMP-14 control, respectively.

[00299] Example 33 – Osteoinductive Potential of Modified rhBMP-14 with or without a Protease

[00300] Dispase and rhBMP-14 (PeproTech, Inc.) were mixed in microcentrifuge tubes at a molarity ratio of between about 1:100 and about 100:1 and incubated at 37 °C for 10-60 minutes. The mixture was purified with reverse phase HPLC using a C-18 column. The HPLC purified modified rhBMP-14 was used to test the effect of modified rhBMP-14 on osteogenic potential of C2C12 myoblasts.

[00301] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. The purified modified rhBMP-14 alone, or the same concentration of purified modified rhBMP-14 with the addition of dispase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of modified rhBMP-14) were used as a negative control.

[00302] After 3 days incubation at 37 °C, 5% CO₂, the AP assay was performed as described above. As shown in Figure 16, the alkaline phosphatase activity in the cells induced by the purified modified rhBMP-14 in combination with dispase was about 235% higher than that induced by the purified modified rhBMP-14 alone.

[00303] Example 34 – Osteoinductive Potential of Modified rhBMP-14 with or without an additional Protease

[00304] C2C12 cells were cultured as described above. Non-purified modified rhBMP-14 (1 ug/mL), or the same concentration of non-purified modified rhBMP-14 with addition of dispase or collagenase were introduced into wells of the C2C12 cell. The AP assay was performed as described above. As shown in Figure 17, the alkaline phosphatase activity in the cells induced by the non-purified modified rhBMP-14 in combination with dispase or collagenase were about 120% and 45% higher than that induced by the non-purified modified rhBMP-14 alone, respectively.

[00305] Example 35 – Osteoinductive potential of mature rhBMP-14 with the addition of protease

[00306] C2C12 cells (ATCC CRL-1772) were seeded at a density of 25,000 cells/cm² in 24-well plates on day one. The mature rhBMP-14 (1 µg/mL, PeproTech, Inc.), or the same concentration of mature rhBMP-14 with the addition of dispase or collagenase (molarity ratio between about 1:100 and about 100:1) was introduced into wells of the C2C12 cells. C2C12 cells cultured in media alone (without addition of rhBMP-14) were used as a negative control.

[00307] After 3 days incubation at 37 °C, 5% CO₂, the AP assay was performed as described above. The results showed that the alkaline phosphatase activities in cells induced by the mature rhBMP-14 in combination with either dispase or collagenase was about 8-80 times and 53 – 150% higher than that induced by the mature rhBMP-14 without addition of protease, respectively.

[00308] Example 36 – Effect of Modified rhBMP-14 on tendon-specific gene expression of human adipose-derived stem cells

[00309] Human adipose-derived stem cells (hASC) were isolated from fat tissue of a human donor with research consent. Cells were propagated until the 5th passage and seeded at 5,000 cells/cm² in 6-well plates. After one day, the culture media was changed to the differentiation media that contains rhBMP-14 control (100ng/mL) or dispase-modified rhBMP-14 (100ng/mL) prepared as shown above.

[00310] The cells were cultured in differentiation media for a total of 12 days and the media was changed every 3 days. Cells were collected at different time points and total RNA was extracted and followed by reverse transcription using Applied Biosystems RNA-to-cDNA kit. The tendon-specific gene, scleraxis (SCX), was amplified and quantified using TaqMan Gene Expression Assay kit from Applied Biosystems, Inc. The relative expression (ΔC_t) of the target gene was normalized to GAPDH endogenous gene expression and the fold change ($2^{-\Delta\Delta C_t}$) was calculated relative to the hASC control. The SCX gene expressions in cells cultured with dispase-modified rhBMP-14 were about 30% and 39% higher than that in cells cultured with the rhBMP-14 control on day 9 and day 12, respectively.

[00311] Example 37 – Stability of Modified rhBMP-2 with or without a Protease in Cell Culture

[00312] C2C12 cells were cultured, and purified and non-purified modified rhBMP-2 were prepared as described herein. The purified modified rhBMP-2, the non-purified modified rhBMP-2, or the untreated mature rhBMP-2 at the same concentration was introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of rhBMP-2 or modified rhBMP-2 were used as a negative control. After incubation at 37 °C, 5% CO₂ for different durations (24 hr, 48 hr, or 72 hr), spent culture media were collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates were incubated at 37°C, 5% CO₂ for another 3 days. Cells from the original 24-well

plates and new 24-well plates were collected at the end of each culture period and cell lysates were prepared for AP assays and BCA total protein assays.

[00313] For the control groups wherein C2C12 cells were cultured for three days in untreated rhBMP-2 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity reduced 96%, 98%, and 99% respectively as compared to that of cells cultured for three days with fresh rhBMP-2 media. For the experimental groups in which C2C12 cells were cultured for three days in purified modified rhBMP-2 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity reduced 41%, 75%, and 99% respectively as compared to that of cells cultured for three days with fresh purified modified rhBMP-2 media. For the experimental groups in which C2C12 cells were cultured for three days in non-purified modified rhBMP-2 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity was maintained at a similar or higher level as compared to that of cells cultured for three days with fresh non-purified modified rhBMP-2 media. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-2 in culture media.

[00314] Example 38 – Stability of Modified rhBMP-5 with or without a Protease in Cell Culture

[00315] C2C12 cells were cultured. Purified dispase-modified rhBMP-5 and purified collagenase-modified rhBMP-5 were prepared as described above. The purified dispase-modified rhBMP-5, the purified collagenase-modified rhBMP-5, the purified modified rhBMP-5 with the addition of either dispase or collagenase, or the untreated mature rhBMP-5 at the same concentration were introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of rhBMP-5 or modified rhBMP-5 were used as a negative control. Cells cultured with either dispase or collagenase were used as enzyme negative controls. After incubation at 37 °C, 5% CO₂ for different durations (24 hr, 48 hr, or 72 hr), spent culture media were collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates were incubated at 37°C, 5% CO₂ for another 3 days. Cells from the original 24-well plates and new 24-well plates were collected at the end of each culture period, and cell lysates were prepared for AP assays and BCA total protein assays.

[00316] The enzyme negative control groups containing either dispase or collagenase showed similar alkaline phosphatase activity as the negative control without any addition of rhBMP5 or modified rhBMP5. For the control groups wherein C2C12 cells were cultured for three days in untreated mature rhBMP-5 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity was reduced as compared to that of cells cultured for three days with fresh rhBMP-5 media by 82%, 92% and 94%, respectively.

[00317] For the experimental groups in which C2C12 cells were cultured for three days with the spent media of purified dispase-modified rhBMP-5 collected after 24, 48, and 72hrs, the alkaline phosphatase activity increased about 14%, reduced 10%, and 14% respectively as compared to that of cells cultured for three days with fresh purified dispase-modified BMP5 media.

[00318] For the experimental groups in which C2C12 cells were cultured for three days with the spent media of purified dispase-modified rhBMP-5 and additional dispase collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity reduced 4%, 16%, and 4% respectively as compared to that of cells cultured for three days with fresh purified dispase-modified rhBMP-5 with the addition of dispase media.

[00319] For the experimental groups in which C2C12 cells were cultured for three days in purified collagenase-modified rhBMP-5 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity was maintained at similar or higher level as compared to that of cells cultured for three days with fresh purified collagenase-modified rhBMP-5 media. For the experimental groups in which C2C12 cells were cultured for three days in purified collagenase-modified rhBMP-5 with the addition of dispase or collagenase spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity was also maintained at similar or higher level as compared to that of cells cultured for three days with fresh purified collagenase-modified rhBMP-5 with the addition of dispase or collagenase media.

[00320] For the experimental groups in which C2C12 cells were cultured for three days with the spent media of purified dispase-modified rhBMP-5 and additional collagenase collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity reduced 19%, 11%, and 37% respectively as compared to that of cells cultured for three days with fresh purified dispase-modified rhBMP-5 with addition of collagenase media. Therefore, the modified rhBMP-5 showed higher stability than the unmodified rhBMP-5.

[00321] Example 39 – Stability of Modified rhBMP-13 with or without a Protease in Cell Culture

[00322] C2C12 cells were seeded and cultured for one day. The same quantity of non-modified mature rhBMP-13 or dispase-modified rhBMP-13 without purification was introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of the non-modified rhBMP-13 or modified rhBMP-13 was used as a negative control. After incubation at 37 °C, 5% CO₂ for different durations (24 hr, 48 hr, or 72 hr), spent culture media were collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates were incubated at 37°C, 5% CO₂ for 3 days. Cells from the original 24-well plates and new 24-well plates were collected at the end of each culture period, and cell lysates were prepared for AP assays and BCA total protein assays.

[00323] For the experimental groups in which C2C12 cells were cultured for three days in non-purified disperse-modified rhBMP-13 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activities were 44, 46, and 30 times higher than those of cells cultured for three days with non-modified rhBMP-13 spent media collected after 24 hrs, 48 hrs, and 72 hrs, respectively.

[00324] Example 40 – Stability of Modified rhBMP-14 with or without a Protease in Cell Culture

[00325] C2C12 cells were seeded and cultured for one day. The same quantity of non-modified mature rhBMP-14 or disperse-modified rhBMP-14 without purification was introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of the non-modified rhBMP-14 or modified rhBMP-14 was used as a negative control. After incubation at 37 °C, 5% CO₂ for different durations (24 hr or 48 hr), spent culture media were collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates were incubated at 37°C, 5% CO₂ for 3 days. Cells from the original 24-well plates and new 24-well plates were collected at the end of each culture period, and cell lysates were prepared for AP assays and BCA total protein assays.

[00326] For the experimental groups in which C2C12 cells were cultured for three days in non-purified disperse modified rhBMP-14 spent media collected after 24 hrs or 48 hrs, the alkaline phosphatase activities were 7.5% and 103% higher than those of cells cultured for three days with non-modified rhBMP-14 spent media collected after 24 hrs and 48 hrs, respectively.

[00327] Example 41 – Stability of Modified rhBMPs- 4 and 7 with or without a Protease in Cell Culture

[00328] C2C12 cells are cultured, and purified and non-purified modified rhBMP-4 and 7 are prepared as described above. Each of the purified modified rhBMP-4 and 7, the non-purified modified rhBMP-4 and 7, and the untreated mature rhBMP-4 and 7 at the same concentration is introduced into each well of the C2C12 cell seeded 24-well plate on day two. C2C12 cells cultured without addition of rhBMP- 4 and 7 or modified rhBMP-4 and 7 are used as a negative control. After incubation at 37 °C, 5% CO₂ for different durations (24 hr, 48 hr, or 72 hr), spent culture media are collected from these original 24-well plates and transferred into new 24-well plates containing fresh C2C12 cells seeded the previous day, and the new 24-well plates are incubated at 37°C, 5% CO₂ for another 3 days. Cells from the original 24-well plates and new 24-well plates are collected at the end of each culture period, and cell lysates are prepared for AP assays and BCA total protein assays.

[00329] For the control groups wherein C2C12 cells are cultured for three days in untreated rhBMP-4 or 7 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity is reduced as compared to that of cells cultured for three days with fresh rhBMP-4 or 7 media, respectively. For the experimental groups in which C2C12 cells are cultured for three days in purified modified

rhBMP-4 or 7 spent media collected after 24 hrs, 48 hrs, and 72 hrs, the alkaline phosphatase activity is also reduced less than for the control group. For the experimental groups in which C2C12 cells are cultured for three days in non-purified modified rhBMP-4 or 7 spent media collected after 24 hrs, 48 hrs, and 72 hrs, however, the alkaline phosphatase activity is maintained at a similar or higher level as compared to that of cells cultured for three days with fresh non-purified modified rhBMP-4 or 7 media, respectively. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-4 or 7 in culture media.

[00330] Example 42 – Stability of Modified rhBMP-2 with or without a Protease on Shelf

[00331] Purified and non-purified modified rhBMP-2 were prepared as described herein. Aliquots of the purified and non-purified modified rhBMP-2 at the same volume and concentration were incubated at 37°C for different durations (2 days to 2 weeks). After each time point, one aliquot from each group was transferred into -20°C freezer until the last time point was completed. The aliquots of purified modified rhBMP-2 or non-purified modified rhBMP-2 from different time points were introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-2 or collagenase were used as a negative control. After 3 days incubation at 37 °C, 5% CO₂, the AP assay as described above was performed.

[00332] The alkaline phosphatase activity of cells cultured with purified modified rhBMP-2 on shelf (37 °C) for 7 days or 14 days reduced 94% and 98% respectively as compared to that of cells cultured with purified modified rhBMP-2 on shelf (37 °C) for 2 days. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-2 on shelf (37 °C) for 7 days or 14 days reduced 7% and 6% respectively as compared to that of cells cultured with non-purified modified rhBMP-2 on shelf (37 °C) for 2 days. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-2 on shelf.

[00333] Example 43 – Stability of Modified rhBMP-5 with or without a Protease on Shelf

[00334] Purified modified rhBMP-5 was prepared as described in Example 11 above. Aliquots of the purified disperse-modified rhBMP-5, the purified disperse-modified rhBMP-5 with the addition of disperse or collagenase, the purified collagenase-modified rhBMP-5, or the untreated mature rhBMP-5 at the same volume and concentration were incubated at 37°C for different durations (1 day to 7 days). After each time point, one aliquot from each group was transferred into -20°C freezer until the last time point was completed. All the aliquots were thawed at the same time and were introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-5 or modified rhBMP-5 were used as a negative control. After 3 days incubation at 37 °C, 5% CO₂, the AP assay as described above was performed.

[00335] The alkaline phosphatase activity of cells cultured with purified dispase-modified rhBMP-5 on shelf (37 °C) for 1 day was reduced about 99% as compared to that of cells cultured with fresh purified modified rhBMP-5, but was about 99 times higher than the alkaline phosphatase activity of the untreated mature rhBMP-5 control after 1 day of incubation. The alkaline phosphatase activity of cells cultured with purified collagenase-modified rhBMP-5 on shelf (37 °C) for 3 days or 7 days was reduced 27% and 97% respectively as compared to that of cells cultured with fresh purified collagenase-modified rhBMP-5. However, the alkaline phosphatase activity of cells cultured with purified dispase-modified rhBMP-5 with the addition of collagenase on shelf (37 °C) for 1 day, 3 days, or 7 days was reduced 17%, 36%, and 61% respectively as compared to that of cells cultured with fresh purified dispase-modified rhBMP-5 with the addition of collagenase. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-5 on shelf.

[00336] In addition, the alkaline phosphatase activity of cells cultured with purified dispase-modified rhBMP-5 with the addition of collagenase on shelf (37 °C) for 1 day, 3 days, or 7 days was 1460 times, 990 times, and 603 times higher than that of cells cultured with non-modified mature rhBMP-5 after 1 day, 3 day, and 7 day shelf-life, respectively.

[00337] Example 44 – Stability of Modified rhBMPs- 4 and 7 with or without a Protease

[00338] Purified and non-purified modified rhBMP-4 and 7 are prepared as described in Examples above. Aliquots of each of the purified and non-purified modified rhBMP-4 or 7 at the same volume and concentration are incubated at 37°C for different durations (2 days to 2 weeks). After each time point, one aliquot from each group is transferred into -20°C freezer until the last time point is completed. The aliquots of each of the purified modified rhBMP-4 or 7, or non-purified modified rhBMP-4 or 7 from different time points are introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-4 or 7 or collagenase are used as a negative control. After 3 days incubation at 37 °C, 5% CO₂, the AP assay as described above is performed.

[00339] The alkaline phosphatase activity of cells cultured with purified modified rhBMP-4 or 7 on shelf (37 °C) for 7 days or 14 days is reduced as compared to that of cells cultured with purified modified rhBMP-4 or 7 on shelf (37 °C) for 2 days. The alkaline phosphatase activity of cells cultured with non-purified modified rhBMP-4 or 7 on shelf (37 °C) for 7 days or 14 days is reduced less than that of cells cultured with purified modified rhBMP-4 or 7, respectively. This demonstrates that the presence of a protease can further enhance the stability of modified BMP-4 and 7 on shelf.

[00340] Example 45 – Stability of Modified rhBMP13 with or without a Protease on shelf

[00341] The same amount of rhBMP13 without treatment and rhBMP13 with dispase or collagenase treatment but without purification were prepared and incubated at 37°C for different durations (1 day

to 10days). After each time point, one aliquot from each group was transferred into -20°C freezer until the last time point was completed. All aliquots were thawed at the same time and were introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-13 or modified rhBMP-13 were used as a negative control. After 3 days incubation at 37 °C, 5% CO₂, the AP assay as described above was performed.

[00342] The alkaline phosphatase activity in cells cultured with non-purified collagenase modified rhBMP-13 stored at 37 °C for 3 days or 10 days reduced 32% and 52%, respectively, as compared to that in cells cultured with fresh non-purified collagenase modified rhBMP-13 without storage; however, they were still 291% and 174% higher than that of cells cultured with non-modified rhBMP-13 stored at 37 °C for 3 days or 10 days. The alkaline phosphatase activity in cells cultured with non-purified disperse modified rhBMP-13 (stored at 37°C) for 3 days or 10 days were 79% and 27% higher than that of cells cultured with non-purified rhBMP-13 (stored at 37°C) for 3 days or 10 days.

[00343] Example 46 – Stability of Modified rhBMP14 with or without a Protease on shelf

[00344] The same amount of rhBMP14 without treatment and rhBMP14 with collagenase treatment but without purification were prepared and incubated at 37°C for different durations (1 day to 10days). After each time point, one aliquot from each group was transferred into -20°C freezer until the last time point was completed. The aliquots of non-modified rhBMP-14 or non-purified collagenase modified rhBMP-14 from different time points were introduced into each well of the C2C12 cell cultured as described above. C2C12 cells cultured without the addition of rhBMP-14 or modified rhBMP-14 were used as a negative control. After 3 days incubation at 37 °C, 5% CO₂, the AP assay as described above was performed.

[00345] The alkaline phosphatase activity of cells cultured with non-purified collagenase modified rhBMP-14 on shelf (37 °C) for 3 days or 7 days only reduced 11% and 12.7% respectively as compared to that of cells cultured with fresh non-purified collagenase modified rhBMP-14, while the alkaline phosphatase activity of cells cultured with non-modified rhBMP-14 on shelf (37 °C) for 3 days or 7 days reduced 25% and 39% respectively as compared to that of cells cultured with fresh non-modified rhBMP-14.

What is Claimed is:

1. A method of promoting differentiation of cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising treating the cells with a peptide selected from the group consisting of
 - a) a peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27, or 28
 - b) a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34 or 35,
 - c) a peptide of 137 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27, or 28,
 - d) a peptide of 119 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:34 or 35, and
 - e) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27, 28, 34 or 35,wherein each of peptides (a)-(e) has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.
2. The method of claim 1, wherein the cells are progenitor cells or adult stem cells.
3. The method of claim 2, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.
4. The method of claim 1, wherein the cells are pluripotent stem cells.
5. A method of promoting osteogenesis, chondrogenic, or ligament/tendon differentiation of cells in a tissue, the method comprising treating the tissue with a peptide selected from the group consisting of
 - a) a peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28,

- b) a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34 or 35,
- c) a peptide of 137 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28,
- d) a peptide of 119 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:34 or 35, and
- e) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27, 28, 34 or 35,

wherein each of peptides (a)-(e) has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.

- 6. The method of claim 5, wherein the osteogenic activity of cells in the treated tissue is greater than the osteogenic activity of cells in untreated tissue.
- 7. The method of claim 5, wherein the tissue is bone, cartilage, or connective tissue.
- 8. An isolated peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.
- 9. The isolated peptide of claims 8, wherein the amino acid sequence comprises the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.
- 10. The isolated peptide of claim 9, wherein the peptide consists of the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.
- 11. A fusion protein comprising (a) the peptide of any of claims 8-10, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO:40, 41, 42, 43, 44, 45, 46, 47 or 48.
- 12. A composition comprising the peptide of claim 8 and at least one protease.
- 13. The composition according to claim 12, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

14. A method of making the peptide of any one of claims 8-10 comprising contacting mature BMP-5 with a protease under conditions that promote protein cleavage and harvesting the peptide.
15. The method of claim 14, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.
16. A vector encoding the peptide of any one of claims 8-10.
17. A host cell comprising the vector of claim 16.
18. A method of making the peptide of any one of claims 8-10, comprising culturing a host cell under conditions suitable for protein expression and harvesting the protein, wherein the host cell comprises a vector that encodes said peptide.
19. A pharmaceutical composition comprising the peptide of any one of claims 8-10.
20. A method of increasing a growth factor activity, the method comprising treating a cell with at least one protease and the growth factor, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27 or 28, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.
21. The method according to claim 20, wherein the peptide is selected from the group consisting of
 - a) a peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28,
 - b) a peptide of 137 residues or less, said peptide comprising the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28, and
 - c) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28.
22. The method according to claim 20, wherein the peptide is a fusion protein comprising (a) the peptide comprising the amino acid sequence of SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27 or 28, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO: 45.
23. The method according to claim 20, wherein the composition comprises two or more proteases.

24. The method according to claim 20, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.
25. The method according to claim 24, wherein the at least one protease is collagenase, dispase or cathepsin.
26. The method according to claim 20, wherein the cells are progenitor cells or adult stem cells.
27. The method according to claim 26, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.
28. The method according to claim 26, wherein the progenitor cells or the adult stem cells are derived from pluripotent stem cells.
29. The method according to claim 20, wherein the cells are pluripotent stem cells.
30. An isolated peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.
31. The isolated peptide of claims 30, wherein the amino acid sequence comprises the amino acid sequence of SEQ ID NO:34.
32. The isolated peptide of claim 31, wherein the peptide consists of the amino acid sequence of SEQ ID NO:34.
33. A fusion protein comprising (a) the peptide of any of claims 30-32, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO:54.
34. A composition comprising the peptide of claim 30 and at least one protease.
35. The composition according to claim 34, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.

36. A method of making the peptide of any one of claims 30-32 comprising contacting mature BMP-13 with a protease under conditions that promote protein cleavage and harvesting the peptide.
37. The method of claim 36, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.
38. A vector encoding the peptide of any one of claims 30-32.
39. A host cell comprising the vector of claim 38.
40. A method of making the peptide of any one of claims 30-32, comprising culturing a host cell under conditions suitable for protein expression and harvesting the protein, wherein the host cell comprises a vector that encodes said peptide.
41. A pharmaceutical composition comprising the peptide of any one of claims 30-32.
42. A method of increasing a growth factor activity, the method comprising treating a cell with at least one protease and the growth factor, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 34, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.
43. The method according to claim 42, wherein the peptide is selected from the group consisting of
 - a) a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 34,
 - b) a peptide of 119 residues or less, said peptide comprising an amino acid sequence of SEQ ID NO: 34, and
 - c) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO: 34.
44. The method according to claim 42, wherein the peptide is a fusion protein comprising (a) the peptide comprising the amino acid sequence of SEQ ID NO: 33, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO: 54.

45. The method according to claim 42, wherein the composition comprises two or more proteases.
46. The method according to claim 42, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.
47. The method according to claim 46, wherein the at least one protease is collagenase, dispase or cathepsin.
48. The method according to claim 42, wherein the cells are progenitor cells or adult stem cells.
49. The method according to claim 48, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle
50. The method according to claim 48, wherein the progenitor cells or the adult stem cells are derived from pluripotent stem cells.
51. The method according to claim 42, wherein the cells are pluripotent stem cells.
52. An isolated peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.
53. The isolated peptide of claims 52, wherein the amino acid sequence comprises the amino acid sequence of SEQ ID NO:35.
54. The isolated peptide of claim 53, wherein the peptide consists of the amino acid sequence of SEQ ID NO:35.
55. A fusion protein comprising (a) the peptide of any of claims 52-54, and (b) a second peptide, wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO:55.
56. A composition comprising the peptide of claim 52 and at least one protease.

57. The composition according to claim 56, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.
58. A method of making the peptide of any one of claims 52-54 comprising contacting mature BMP-14 with a protease under conditions that promote protein cleavage and harvesting the peptide.
59. The method of claim 58, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.
60. A vector encoding the peptide of any one of claims 52-54.
61. A host cell comprising the vector of claim 60.
62. A method of making the peptide of any one of claims 52-54, comprising culturing a host cell under conditions suitable for protein expression and harvesting the protein, wherein the host cell comprises a vector that encodes said peptide.
63. A pharmaceutical composition comprising the peptide of any one of claims 52-54.
64. A method of increasing a growth factor activity, the method comprising treating a cell with at least one protease and the growth factor, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.
65. The method according to claim 64, wherein the peptide is selected from the group consisting of
- d) a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 35,
 - e) a peptide of 119 residues or less, said peptide comprising an amino acid sequence of SEQ ID NO: 35, and
 - f) a peptide with an amino acid sequence consisting of the amino acid sequence of SEQ ID NO: 35.
66. The method according to claim 64, wherein the peptide is a fusion protein comprising (a) the peptide comprising the amino acid sequence of SEQ ID NO: 35, and (b) a second peptide,

wherein the second peptide comprises an amino acid sequence that is less than 70% identical to the amino acid sequence of SEQ ID NO: 55.

67. The method according to claim 64, wherein the composition comprises two or more proteases.
68. The method according to claim 64, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.
69. The method according to claim 68, wherein the at least one protease is collagenase, dispase or cathepsin.
70. The method according to claim 64, wherein the cells are progenitor cells or adult stem cells.
71. The method according to claim 70, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle
72. The method according to claim 70, wherein the progenitor cells or the adult stem cells are derived from pluripotent stem cells.
73. The method according to claim 64, wherein the cells are pluripotent stem cells.
74. A composition comprising at least one protease and a growth factor, wherein the growth factor is (i) a bone morphogenic protein (BMP) or (ii) an isolated peptide comprising an amino acid sequence at least 92% identical to any of amino acid sequences of SEQ ID NO: 16-35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity.
75. The composition according to claim 74, wherein the growth factor is BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, or BMP-16.
76. The composition according to claim 74, wherein the growth factor comprises an amino acid sequence selected from the group consisting of residue 283-396 of SEQ ID NO: 1, residue 363-472 of SEQ ID NO: 2, residue 293-408 of SEQ ID NO: 3, residue 317-454 of SEQ ID NO: 4, residue 375-513 of SEQ ID NO: 5, residue 293-431 of SEQ ID NO: 6, residue 243-

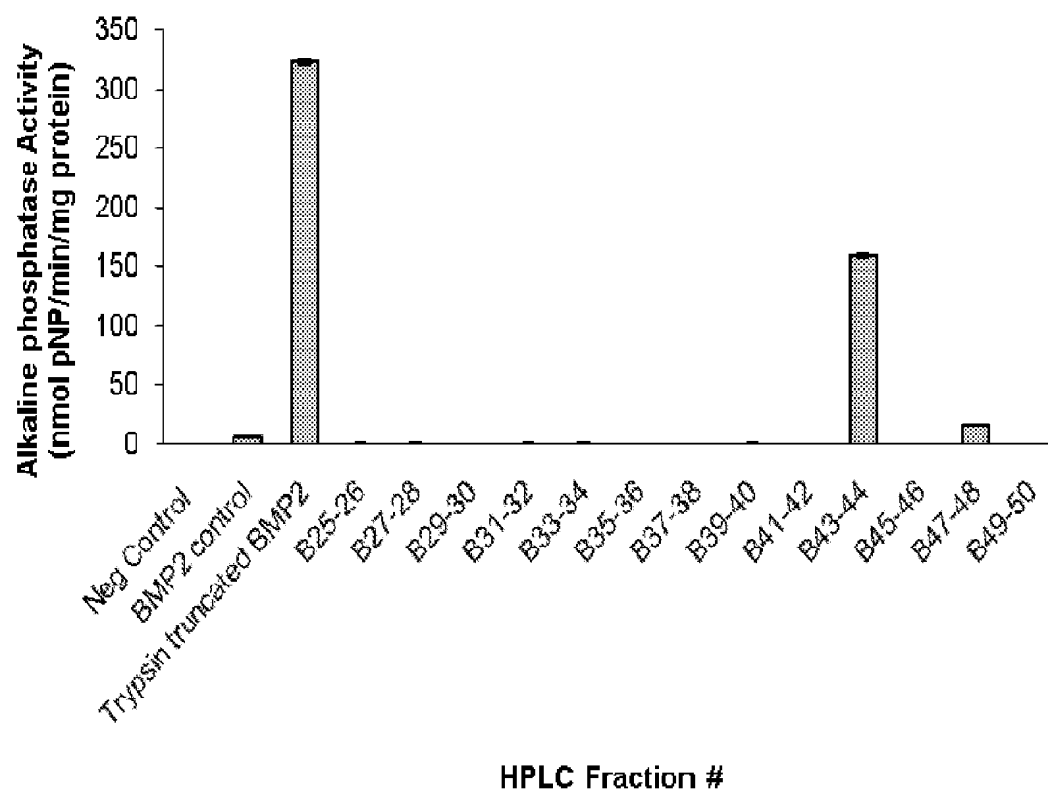
402 of SEQ ID NO: 7, residue 319-429 of SEQ ID NO: 8, residue 316-424 of SEQ ID NO: 9, residue 298-407 of SEQ ID NO: 10, residue 316-461 of SEQ ID NO: 11, residue 336-455 of SEQ ID NO: 12, residue 382-501 of SEQ ID NO: 13, residue 267-392 of SEQ ID NO: 14, and residue 171-280 of SEQ ID NO: 15.

77. The composition according to claim 74, wherein the growth factor comprises an amino acid sequence at least 92% identical to any of amino acid sequences of SEQ ID NO: 16-35.
78. The composition according to claim 76, wherein the peptide is selected from the group consisting of
- a) a first peptide of an isolated peptide of 113 residues or less comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 16, 17, or 18;
 - b) a second peptide of an isolated peptides of 115 residues or less comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO: 19;
 - c) a third peptide of an isolated peptides of 137 residues or less comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27 or 28;
 - d) a fourth peptide of an isolated peptides of 138 residues or less comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO: 29;
 - e) a fifth peptide of an isolated peptides of 138 residues or less comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 30, 31, 32 or 33;
 - f) a sixth peptide of an isolated peptides of 119 residues or less comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 34; and
 - g) a seventh peptide of an isolated peptides of 119 residues or less comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO: 35.
79. The composition according to claim 76, wherein the peptide is selected from the group consisting of
- a) a first peptide of an isolated peptide of 113 residues or less comprising the amino acid sequence of SEQ ID NO: 16, 17, or 18;
 - b) a second peptide of an isolated peptides of 115 residues or less comprising the amino acid sequence of SEQ ID NO: 19;
 - c) a third peptide of an isolated peptides of 137 residues or less comprising the amino acid sequence of SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27 or 28;

- d) a fourth peptide of an isolated peptides of 138 residues or less comprising the amino acid sequence of SEQ ID NO: 29;
 - e) a fifth peptide of an isolated peptides of 138 residues or less comprising the amino acid sequence of SEQ ID NO: 30, 31, 32 or 33;
 - f) a sixth peptide of an isolated peptides of 119 residues or less comprising the amino acid sequence of SEQ ID NO: 34; and
 - g) a seventh peptide of an isolated peptides of 119 residues or less comprising the amino acid sequence of SEQ ID NO: 35.
- 80. The composition according to claim 74, wherein the peptide is selected from the group consisting any of amino acid sequences of SEQ ID NO: 16-35.
 - 81. The composition according to claim 74, wherein the composition comprises two or more proteases.
 - 82. The composition according to claim 74, wherein the at least one protease is selected from the group consisting of collagenase, clostripain, dispase, trypsin, cathepsin, BMP-1 (bone morphogenetic protein-1), MMP-13 (matrix metalloproteinase-13), and a mixture thereof.
 - 83. The composition according to claim 74, wherein the at least one protease is collagenase, dispase or cathepsin.
 - 84. A method of increasing a growth factor activity, the method comprising treating a cell with the composition of claims 80-83.
 - 85. The method according to claim 84, wherein the cells are progenitor cells or adult stem cells.
 - 86. The method according to claim 85, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.
 - 87. The method according to claim 85, wherein the progenitor cells or the adult stem cells are derived from pluripotent stem cells.
 - 88. The method according to claim 84, wherein the cells are pluripotent stem cells.

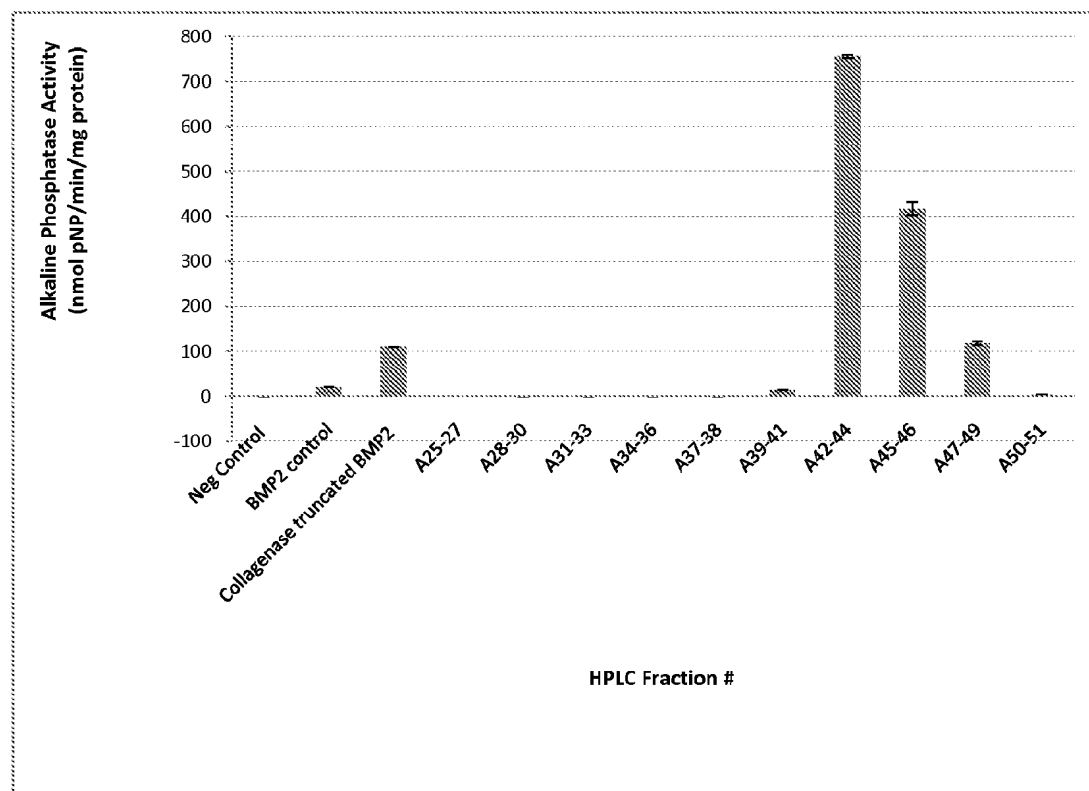
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Figure 1:



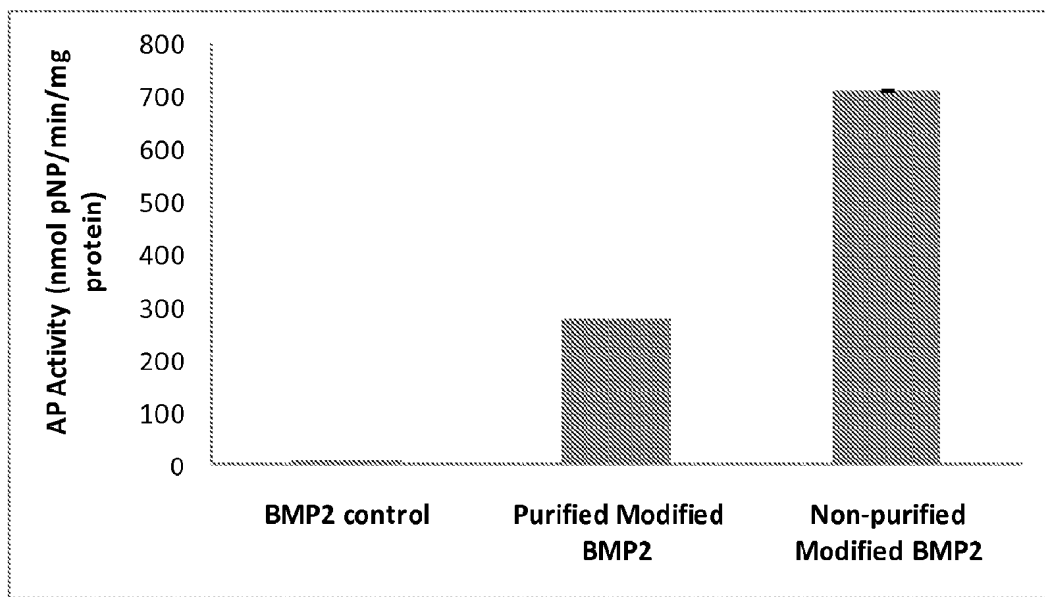
2/17

Figure 2:



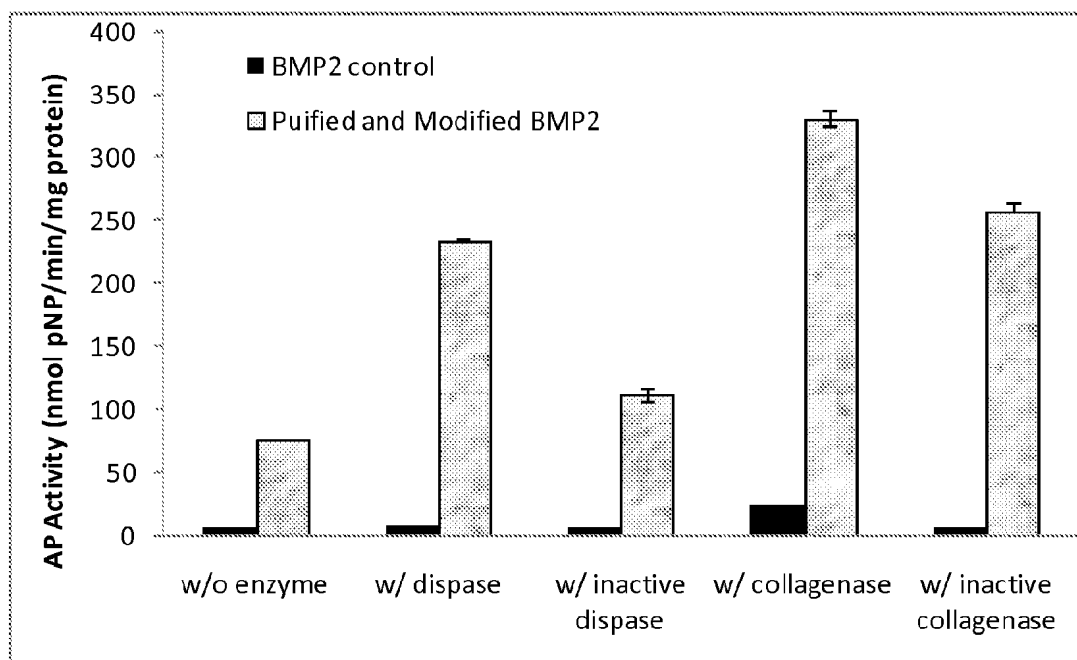
3/17

Figure 3:



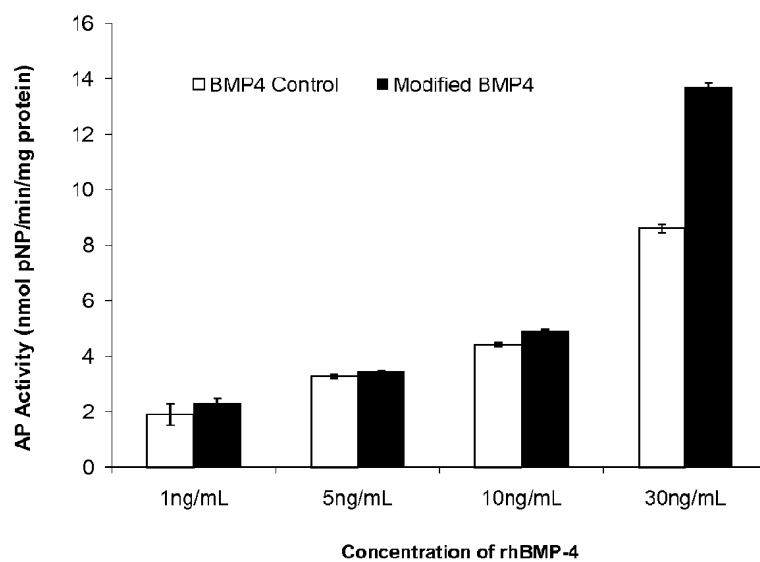
4/17

Figure 4:



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Figure 5:



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Figure 6:

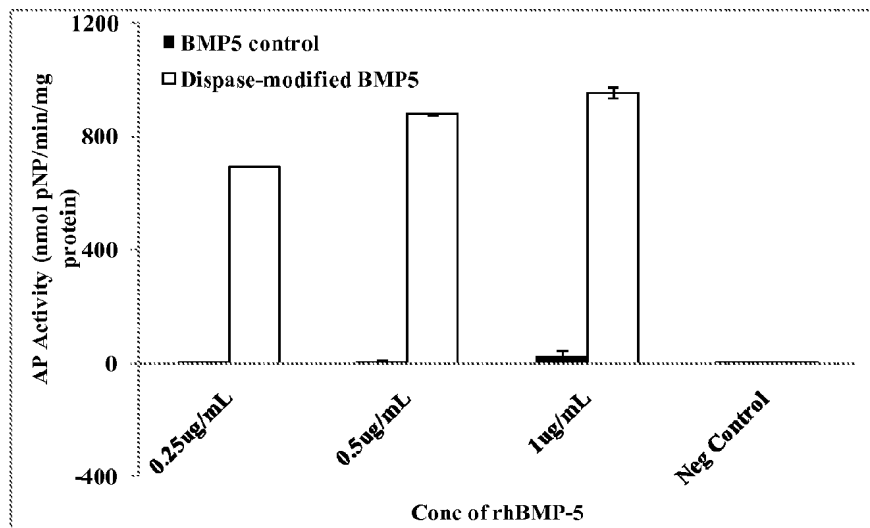
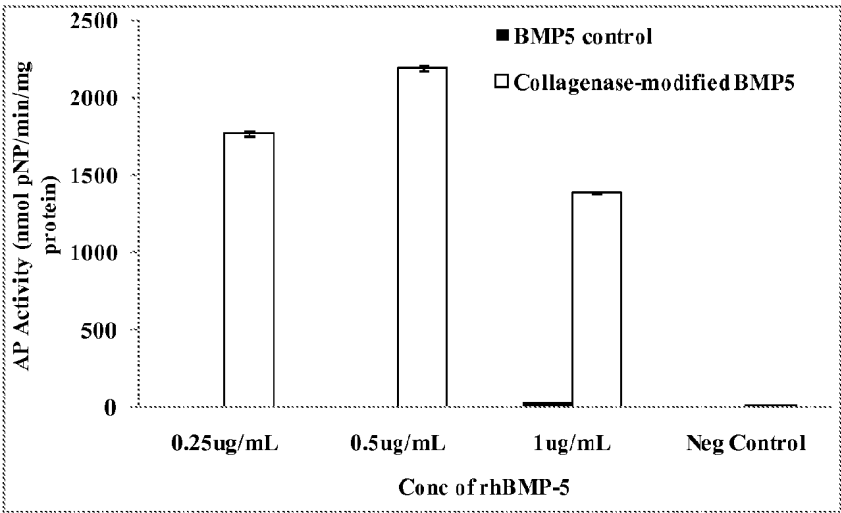
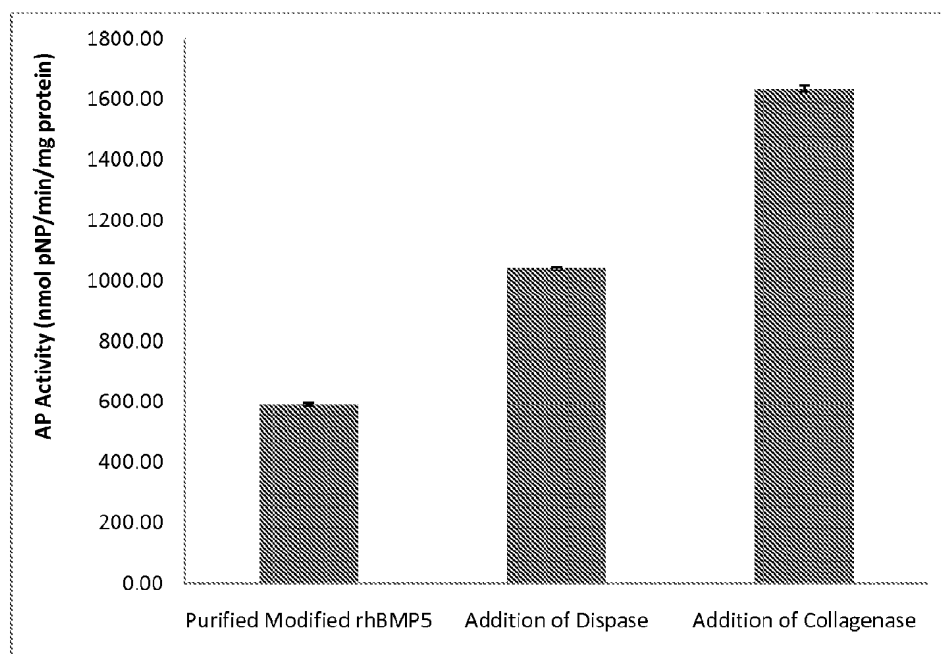


Figure 7:



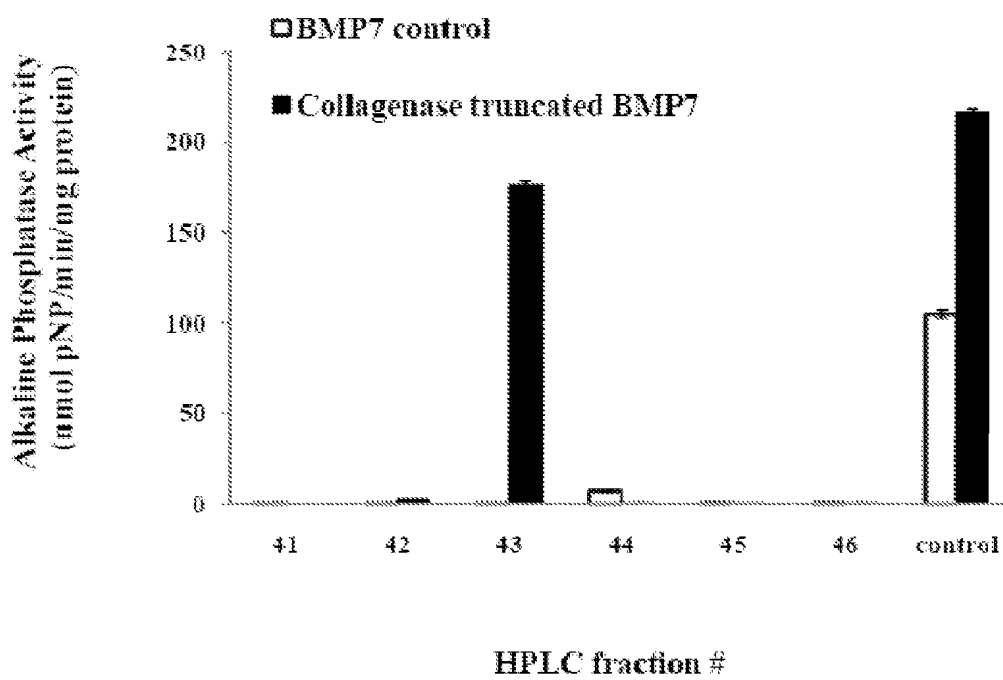
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Figure 8:



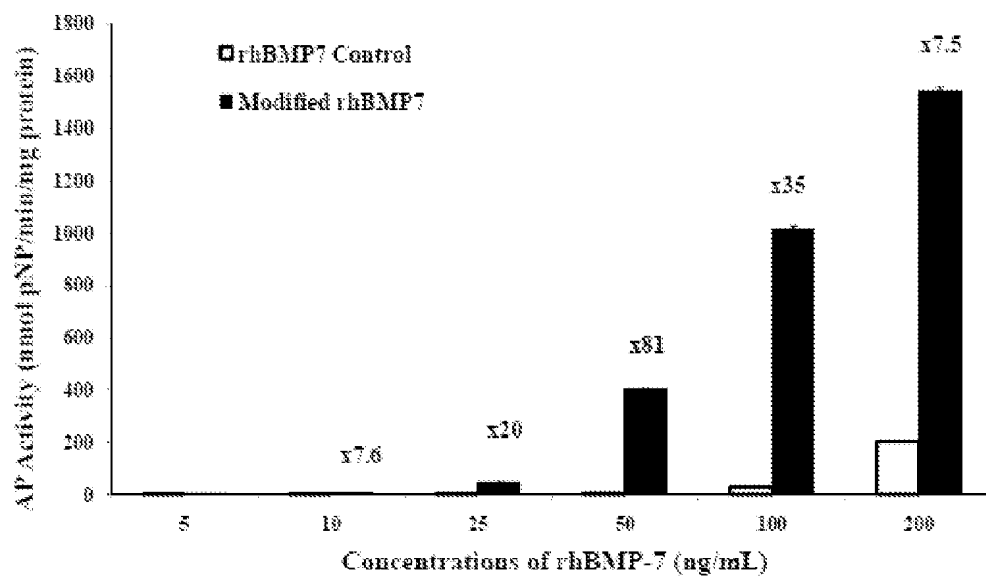
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Figure 9:



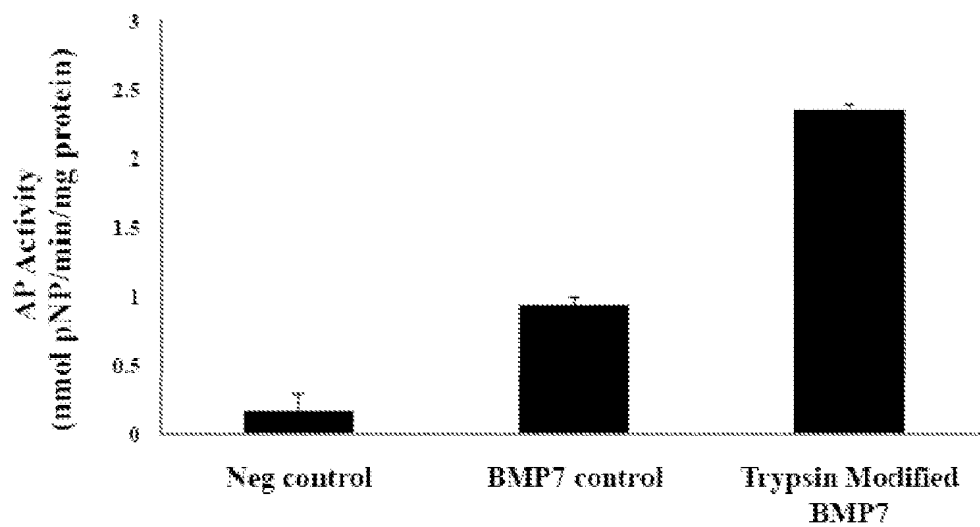
10/17

Figure 10:



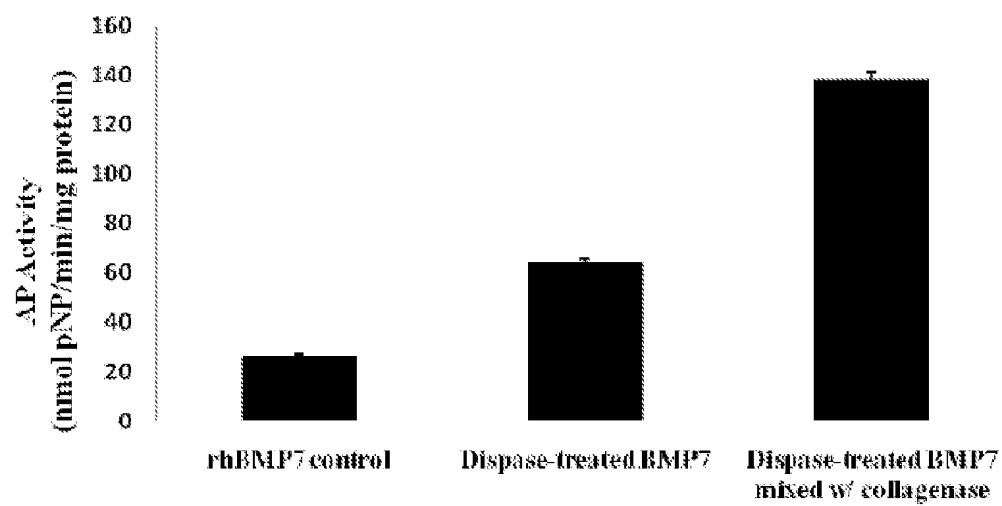
11/17

Figure 11:



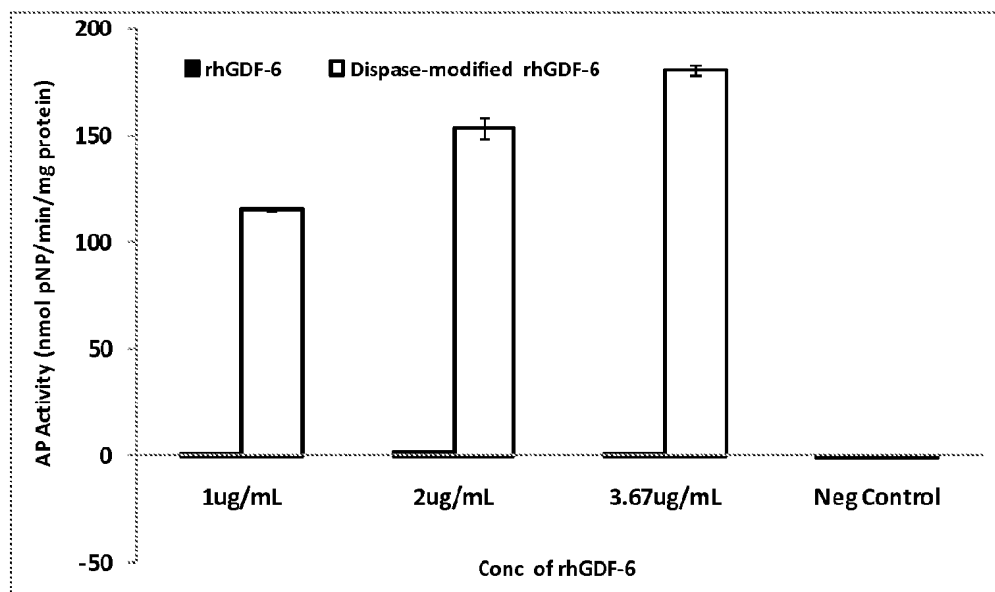
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Figure 12:



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Figure 13:



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Figure 14:

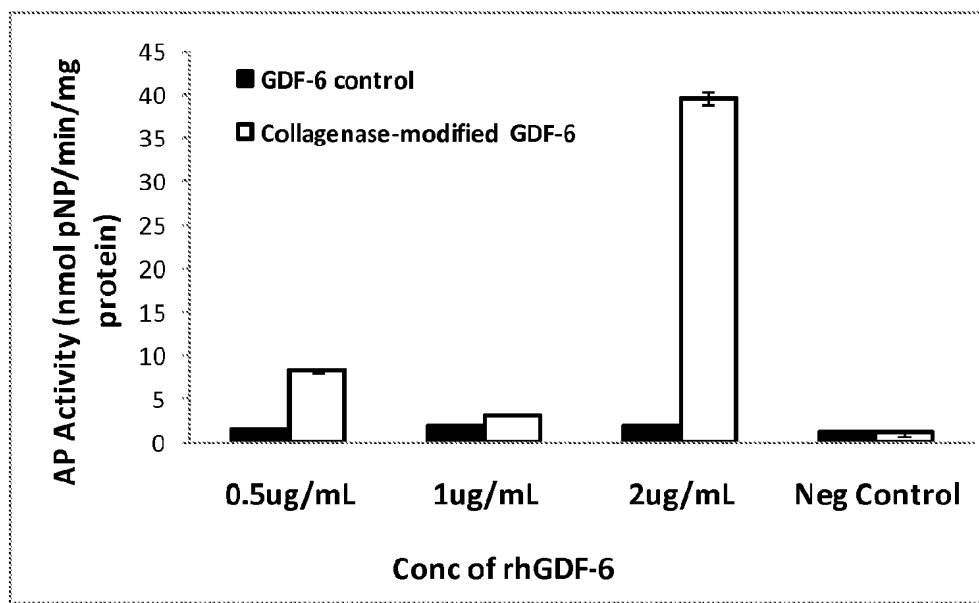
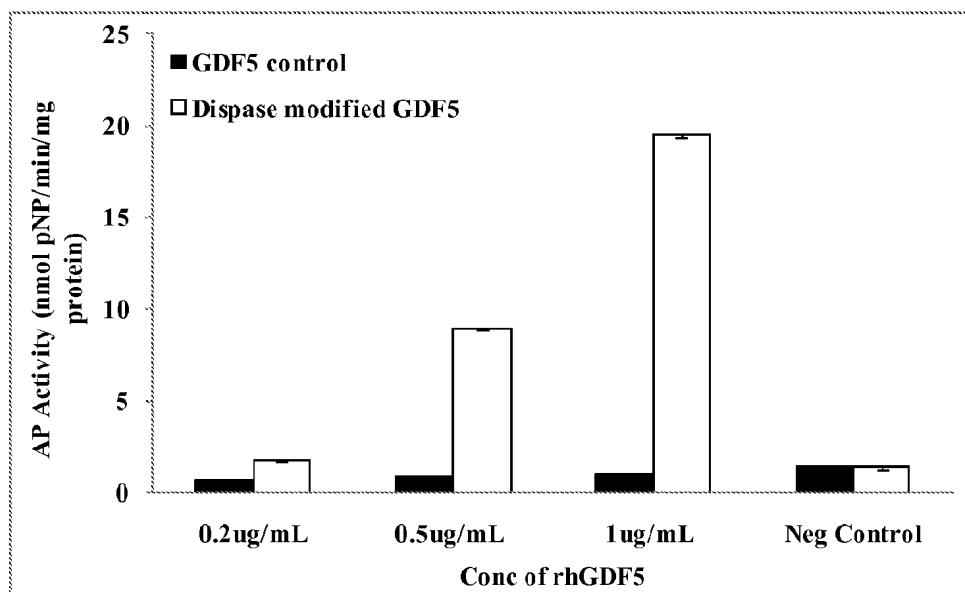
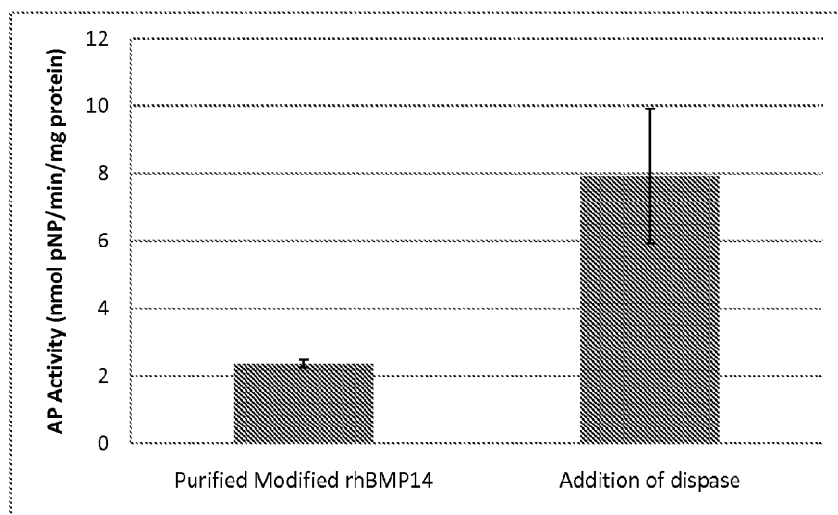


Figure 15:



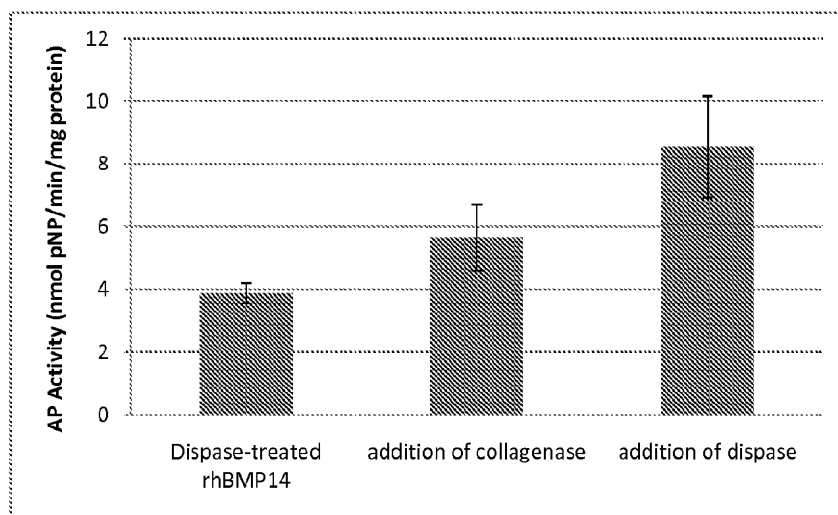
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Figure 16:



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Figure 17:



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/53584

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/077, A61P 19/00 (2012.01)

USPC - 435/377, 435/405, 514/8.8

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 435/377, 435/405, 514/8.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC: 424/93.21; 435/325, 375, 377, 404, 405; 514/8.8, 16.7, 17.1

(keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase; Google; PubMed

Search terms: BMP-5, osteoblast, chondrocyte, ligament, tendon, differentiation, pluripotent, stem cells, SEQ ID NO:20

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2009/0298761 A1 (ENGELMAN) 3 December 2009 (03.12.2009) para [0013], [0025], [0044], [0073], [0083], [0088], [0092], [0103]; SEQ ID NO:13	1-7
Y	US 2001/0018509 A1 (LEE et al.) 30 August 2001 (30.08.2001) para [0004], [0094]; SEQ ID NO:13	1-7
Y	US 2005/0136042 A1 (BETZ et al.) 23 June 2005 (23.06.2005) para [0079], [0091], [0180], [0188]	4
A	WU et al., Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization. J. Bone Miner. Res. April 2002, Vol. 17, No. 4, pages 639-651	1-7
A	US 5,770,444 A (LEE et al.) 23 June 1998 (23.06.1998) entire document	1-7
A	US 7,198,790 A (LEE et al.) 3 April 2007 (03.04.2007) entire document	1-7

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 January 2013 (22.01.2013)

Date of mailing of the international search report

05 FEB 2013

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/53584

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-7, drawn to a method of promoting osteogenesis, chondrogenic, or ligament/tendon differentiation comprising treating the cells with a peptide comprising an amino acid having identity to the amino acid sequence of SEQ ID NO:20.

Group II+: Claims 1-7, drawn to a method of promoting osteogenesis, chondrogenic, or ligament/tendon differentiation comprising treating the cells with a peptide comprising an amino acid sequence having identity to the amino acid sequence of SEQ ID NO:21, 22, 23, 24, 25, 26, 27, 28, 34, or 35. If Applicant elects to have this group searched, Applicant must specify the specific amino acid sequence to be searched. Each unique amino acid sequence constitutes an inventive concept.

---please see continuation on extra sheet---

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos. 1-7, limited to SEQ ID NO:20

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/53584

Continuation of: Box No III Observations where unity of invention is lacking

Group III+: Claim 8-19, drawn to an isolated peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28. If Applicant elects to have this group searched, Applicant must specify the specific amino acid sequence to be searched. Each unique amino acid sequence constitutes an inventive concept.

Group IV+: Claims 20-29, drawn to a method of increasing a growth factor activity, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 20, 21,22,23, 24, 25, 26, 27 or 28. If Applicant elects to have this group searched, Applicant must specify the specific amino acid sequence to be searched. Each unique amino acid sequence constitutes an inventive concept.

Group V: Claims 30-41, drawn to an isolated peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34.

Group VI: Claims 42-51, drawn to a method of increasing a growth factor activity, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 34.

Group VII: Claims 52-63, drawn to an isolated peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:35.

Group VIII: Claims 64-73, drawn to a method of increasing a growth factor activity, wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO: 35.

Group IX: Claims 74-88, drawn to a composition comprising at least one protease and a growth factor

The inventions listed as Groups I, II+, III+, IV+, V, VI, VII, VIII, and IX do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of the claims of each of the Groups are disclosed in the Group descriptions, above.

The shared technical feature of the inventions listed as Groups I and II+ is the method steps comprising treating cells with a peptide set forth in claim 1, wherein each of peptides has osteoinductive, chondroinductive, or ligament/tendon differentiating activity. This shared technical feature fails to provide a contribution over the prior art, as evidenced by US 2009/0298761 A1 to Engelman in view of US 2001/0018509 A1 to Lee et al. (hereinafter 'Lee '509'). Engelman discloses a method of promoting differentiation of cells into chondrocytes, ligament, or tendon (para [0013] - "A class of proteins known as "osteogenic proteins" or "morphogenic proteins" or "morphogens," are competent to act as true bone and cartilage tissue morphogens, able, on their own, to induce the proliferation and differentiation of progenitor cells into functional bone, cartilage, tendon, and/or ligamentous tissue"; para [0073] - "The term "cartilage" refers to a type of connective tissue that contains chondrocytes or chondrocyte-like cells"), the method comprising treating the cells (para [0092] - "contacting the cartilage tissue with the soluble morphogenic protein complex") with a peptide with an amino acid sequence comprising of the amino acid sequence of SEQ ID NO:20 (para [0025] - BMP-5, GDF-6; para [0044] - BMP-5 sequence set forth as SEQ ID NO:13, which is 100% identical to claimed SEQ ID NO:20), wherein the peptide has chondroinductive or ligament/tendon differentiating activity (para [0013]). Although Engelman does not disclose wherein the amino acid sequence comprising SEQ ID NO:20 is 137 residues in length or less, Lee '509 teaches a peptide comprising the amino acid sequence of SEQ ID NO:20 that has a length of 137 residues or less (SEQ ID NO:13 is 118 residues in length and exhibits 100% identity to SEQ ID NO:20). It would have been obvious to one of ordinary skill in the art that the peptide of Lee '509 can be used in the method of Engelman because the peptide in the method of Engelman is a BMP-5 peptide (para [0025]), and the sequence disclosed by Lee '509 is that of the C-terminal region of BMP-5 (para [0094]) and Lee '509 teaches that BMPs can induce de novo cartilage and bone formation (para [0004]). In the absence of a contribution over the prior art, the shared technical feature is not a shared special technical feature.

A further shared technical feature of the inventions listed as Groups I and II+ is the specific amino acid sequences recited therein. Significant structural similarities cannot readily be ascertained among the amino acid sequences. Without significant structural similarities, the amino acid sequences do not have a shared special technical feature. In the absence of a shared special technical feature, the inventions lack unity with one another.

The shared technical feature of the inventions listed as Groups III+ is an isolated peptide of 137 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:20, 21, 22, 23, 24, 25, 26, 27 or 28. This shared technical feature fails to provide a contribution over Lee '509, which discloses a peptide comprising the amino acid sequence of SEQ ID NO:20 that has a length of 137 residues or less (SEQ ID NO:13 is 118 residues in length and exhibits 100% identity to SEQ ID NO:20). Furthermore, significant structural similarities cannot readily be ascertained among the amino acid sequences. Without significant structural similarities, the amino acid sequences do not have a shared special technical feature. In the absence of a shared special technical feature, the inventions lack unity with one another.

The shared technical feature of the inventions listed as Groups II+, V, and VI is a peptide comprising SEQ ID NO:34. The shared technical feature of the inventions listed as Groups II+ and V is a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity. This shared technical feature fails to provide a contribution over the prior art, as evidenced by US 5770444 A to Lee et al. (hereinafter 'Lee '444'), which discloses a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:34 (SEQ ID NO:7 is 119 residues in length and exhibits 100% identity to SEQ ID NO:34), wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity (col 14, ln 9-10 - "SEQ ID NO:7 is the amino acid for the C-terminal sequence of GDF-6"; col 3, ln 19-25 - "The BMP's can induce new bone growth and are effective for the treatment of fractures and other skeletal defects . . . GDF-6 may have similar activities and may be useful in repair of tissue injury caused by trauma or burns for example"). In the absence of a shared special technical feature, the inventions lack unity with one another.

—please see continuation on next extra sheet—

Form PCT/ISA/210 (extra sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/53584

Continuation of: Previous extra sheet (Box No III Observations where unity of invention is lacking)

The shared technical feature of the inventions listed as Groups II+, VII and VIII is a peptide comprising SEQ ID NO: 35. The shared technical feature of the inventions listed as Groups II+ and VII is a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:35, wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity. This shared technical feature fails to provide a contribution over the prior art, as evidenced by US 7198790 A to Lee et al. (hereinafter 'Lee '790'), which discloses a peptide of 119 residues or less, said peptide comprising an amino acid sequence at least 92% identical to the amino acid sequence of SEQ ID NO:35 (SEQ ID NO:13 is 119 residues in length and exhibits 100% identity to SEQ ID NO:35), wherein the peptide has osteoinductive, chondroinductive, or ligament/tendon differentiating activity (col 2, ln 27-28 - "C-terminal sequences of GDF-5 (SEQ ID NO:13)"; col 3, ln 28-33 - "The expression of GDF-5 during embryogenesis and specifically in the precartilaginous mesenchyme associated with early bone formation in the limbs, suggests a variety of applications using the polypeptide, polynucleotide, and antibodies of the invention, related to skeletal development, cartilage differentiation, and cell proliferative diseases."). In the absence of a shared special technical feature, the inventions lack unity with one another.

The shared technical feature of the inventions listed as Groups IV+, VI, VIII and IX is a composition or method comprising at least one protease and a growth factor. The shared technical feature of the inventions listed as Groups IV+, VI, and VIII is method of increasing a growth factor activity, the method comprising treating a cell with at least one protease and a growth factor, wherein the growth factor is a peptide having osteoinductive, chondroinductive, or ligament/tendon differentiating activity. This shared technical feature fails to provide a contribution over Engelman in view of the article entitled "Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization" by Wu et al. (published in J. Bone Miner. Res. April 2002, Vol. 17, No. 4, pages 639-651; hereinafter 'Wu'). Engelman teaches a method comprising treating a cell with at least one growth factor (para [0092] - "contacting the cartilage tissue with the soluble morphogenic protein complex"), wherein the growth factor is a peptide comprising an amino acid sequence of SEQ ID NO:20 (para [0025] - BMP-5; para [0044] - BMP-5 sequence set forth as SEQ ID NO:13, which is 100% identical to claimed SEQ ID NO:20), wherein the peptide has chondroinductive or ligament/tendon differentiating activity (para [0013] - "A class of proteins known as "osteogenic proteins" or "morphogenic proteins" or "morphogens," are competent to act as true bone and cartilage tissue morphogens, able, on their own, to induce the proliferation and differentiation of progenitor cells into functional bone, cartilage, tendon, and/or ligamentous tissue"; para [0073] - "The term "cartilage" refers to a type of connective tissue that contains chondrocytes or chondrocyte-like cells"). Engelman does not teach that the method further comprises treating the cell with at least one protease. Wu teaches that the protease MMP-13 is required for chondrocyte differentiation (abstract). Accordingly, it would have been obvious to one of skill in the art that it would be beneficial to additionally treat the cells with MMP-13 in the method of Engelman, in order to ensure optimal chondrocyte differentiation (and which would inherently increase the morphogenic activity of the growth factor). In the absence of a contribution over the prior art, the shared technical feature is not a shared special technical feature.

A further shared technical feature of the inventions listed as Groups IV+ is the specific amino acid sequences recited therein. Significant structural similarities cannot readily be ascertained among the amino acid sequences. Without significant structural similarities, the amino acid sequences do not have a shared special technical feature. In the absence of a shared special technical feature, the inventions lack unity with one another.

Therefore, the inventions of Groups I, II+, III+, IV+, V, VI, VII, VIII, and IX lack unity with one another because they fail to share a same or corresponding special technical feature.



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(74) Agents: **LEE, H., Janice** et al.; Morgan, Lewis & Bockius LLP, 1111 Pennsylvania Avenue, NW, Washington, DC 20004 (US).

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(54) Title: LIQUEFACTION OF BONE MATRIX

(57) Abstract: The invention relates to methods of preparing a bone matrix solution, a bone matrix implant, and variants thereof. The invention also relates to methods of cell culture using the same. The invention further relates to bone matrix scaffolds comprising one or more bone matrix nanofibers, methods of preparing, and methods of use thereof. The invention also relates to methods of culturing cells and promoting differentiation of stem cells using the same.



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LIQUEFACTION OF BONE MATRIX

Background of the Invention

[0001] The invention relates to methods of preparing a bone matrix solution, a bone matrix implant, and variants thereof. The invention also relates to methods of cell culture using the same.

[0002] The invention further relates to bone matrix scaffolds comprising one or more bone matrix nanofibers, methods of preparing, and methods of use thereof. The invention also relates to methods of culturing cells and promoting differentiation of stem cells using the same.

Summary

[0003] The invention relates to methods of preparing a bone matrix solution comprising dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution.

[0004] The invention also relates to methods of preparing a bone matrix implant with a predetermined shape comprising: dissolving a bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution; exposing the bone solution to conditions sufficient to evaporate an amount of the solvent to form a colloidal gel; and allowing the colloidal gel to harden into the predetermined shape to form the bone matrix implant.

[0005] The invention also relates to methods of preparing a film of bone matrix on a surface comprising: dissolving a bone preparation in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE), and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution; exposing the bone solution to conditions sufficient to evaporate an amount of the solvent to form a colloidal gel; coating the surface with the colloidal gel; and exposing the

coated surface to conditions sufficient to further evaporate an amount of solvent to form a film. In particular, the bone may be demineralized bone or non-demineralized bone.

[0006] The invention also relates to methods of promoting osteogenesis, chondrogenic, or ligament/tendon differentiation of cells, comprising contacting the cells on a surface coated with the bone matrix produced by the method described herein.

[0007] The invention further relates to bone matrix scaffolds comprising one or more bone matrix nanofibers.

[0008] The invention also relates to methods of preparing the bone matrix scaffold, the method comprising dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution at a temperature between about 0 °C and about 22 °C, and electrospinning the bone matrix solution to form the bone matrix scaffold.

[0009] The invention also relates to methods of preparing the bone matrix scaffold, the method comprising dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution at a temperature between about 0 °C and about 22 °C, and neutralizing the bone matrix solution to form the bone matrix scaffold.

[0010] The invention also relates to bone matrix implants produced by the methods of preparing the bone matrix scaffold.

[0011] The invention further relates to methods of cell culture comprising culturing cells on the bone matrix scaffold in condition suitable for the cell culture. The invention also relates to methods of promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on the bone matrix scaffold in conditions suitable for the cell differentiation.

Brief Description of the Drawings

[0012] FIGURE 1 depicts BMP-2 ELISA assay results. An ELISA for BMP-2 confirms that high quantities of BMP-2 are present in the DBM that was dissolved in TFA/HFP and later electrospun. An estimated amount of rhBMP2 was added to the assay based on a serial dilution, with the calculated amount by the ELISA confirming the precision of the assay. The average amount of Gn-HCL extracted

from DBM by established protocols is shown (purple bar) next to the amount extracted from our tested donor (teal bar) using the established guanidine hydrochloride method. Data was normalized and compared to standard controls, with statistical significance determined by a one-way ANOVA with $p < 0.05$.

[0013] FIGURE 2 depicts an exemplary electrospinning apparatus.

[0014] FIGURE 3 depicts electrospun bone nano-features. Representative scanning electron micrographs of electrospun demineralized bone matrix. The architecture of the large demineralized bone matrix chips are shown relative to the pure electrospun demineralized bone matrix (note scale bar size differences), both as uncross-linked and as cross-linked with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and as blended with various ratios of DBM/PCL.

[0015] FIGURE 4 depicts average electrospun bone matrix fiber size. This graph depicts demineralized bone matrix diameter from raw powder and electrospun nanofibers, with variable amounts of polycaprolactone added to the liquefied bone for electrospinning. "Powder" indicates raw ground bone measured across the particle width. The amount of demineralized bone in electrospun fibers are shown as a percent of DBM relative to the amount of PCL in the copolymer blend as a percentage (100=100% DBM, 10=10% DBM and 90% PCL, etc.). Error bars indicate standard deviation and significance is denoted as $*p < 0.05$, as determined by a one way ANOVA followed by Tukey's test for pairwise comparisons. Note: log scale is used on the ordinate axis.

[0016] FIGURE 5 depicts Fourier Transform Infrared Spectroscopy (FTIR). FTIR analysis of demineralized bone matrix and PCL co-polymer blends was performed, confirming homology of electrospun DMB nanofibers to its native DMB powder form. The peaks noted by red arrows have been shown to associate with an intact alpha-helical structure as present in type I collagen.

[0017] FIGURE 6 depicts collagen structural analysis of electrospun demineralized bone. SDS gel show the band pattern of DBM as electrospun alone or with PCL, as compared with pure type I collagen, either with or without pepsin treatment. Size markers (red bands labeled), collagen type I an electrospun DMB are show before and after treatment with pepsin, with the collagen alpha 1 and alpha 2 helices (noted) shown unchanged under the electrospinning conditions.

[0018] FIGURE 7 depicts porosity of electrospun bone matrix scaffolds. The average scaffold porosity was determined by calculating void fraction of dry materials relative to wet material with respect to material density as shown. We show the average scaffold porosity of electrospun DBM in its native/un-

crosslinked form, or as cross-linked by blending with either PCL or EDC, or as cross-linked with EDC with the scaffold weighted to prevent material shrinkage. Overall, electrospun DBMs had average porosity above that of demineralized bone matrix powder.

[0019] FIGURE 8 depicts mesenchymal stem cell attachment on bone matrix scaffolds. Significantly more cells are seen to attach to electrospun DBM as cross-linked with EDC or as blended with PCL as compared to cell left to attach on glass coverslips alone in a cell attachment assay. Cell were grown in serum-free media over the course of 15, 30, 60 and 120 minutes with cell counts of crystal violet stained cells that were quantitated with image J, and verified by optical densitometry of released stain (not shown). The DBM film dissolved rapidly and negatively biased the attachment assay at all time points. Cell attachment was shown to be statistically significantly higher on DBM-EDC, DBM/PCL and PCL relative to attachment on the coverslip alone with cells grown in serum free media as determined by a one-way ANOVA ($P<0.05$).

[0020] FIGURE 9 depicts *in vivo* implants of bone matrix scaffolds. A four-week animal study with implanted EDC cross-linked DBM and DBM/PCL scaffolds was performed using 0.1cc of material implanted into a rat intramuscular muscle pouch assay. H&E stained sections of implanted electrospun DBM constructs indicate minimal immunoreactivity and high cell recruitment and infiltration into electrospun materials. Abundant, dense, loosely-organized layers of mesenchymal-like-cells are seen throughout the electrospun constructs, with a high degree of original scaffold turnover observed, particularly in DBM/PCL sample. Adipogenic differentiation is potentially seen in DBM/EDC samples, with unique interdigitations observed in most DBM/PCL samples at the scaffold/muscle boundary, evocative of a tendon-type structural interface forming.

[0021] FIGURE 10 depicts Alizarin Red S and Toluidine Blue Assay of bone matrix scaffolds. Alizarin red S histology stains confirmed the presence of calcium in adipose stem cell seeded electrospun DBM crosslinked with EDC (A-EDC), with DBM/PCL (A-PCL), with DBM/EDC without cells (A-EDC no ASCs) and of the unprocessed DBM granules (A-RAW) (A). Toluidine blue suggests the formation of new bone from ASCs that are grown in the presence of electrospun DBM (C) vs grown in control ASC media for 3 weeks (B).

[0022] FIGURE 11 depicts osteoinduction capability of bone matrix scaffolds. Uncrosslinked electrospun DBM was cultured with adipose derived stem cells (ASCs) and dental pulp stem cells (DPSCs) to assay the osteoinductive potential of this material. Both ASCs and DPSCs showed expression of alexa488 labeled bone sialo protein I (aka osteopontin) and bone sialo protein II, as seen in

the many cell mounds or nodules that formed throughout the cultures. Cell nuclei are shown counter-labeled with DAPI.

Detailed Description of the Invention

[0023] The invention relates to methods of preparing a bone matrix solution. The method of preparing a bone matrix solution according to some embodiments of the present invention comprises dissolving bone in an anhydrous solvent. The bone includes, but is not limited to, autograft bone, allograft bone, and xenograft bone. Such bone includes any bone from any source, including, but not limited to, bone from a living human donor, bone from a human cadaveric donor, and bone from an animal. The bone may include cortical bone and/or cancellous bone and/or cortico-cancellous bone.

[0024] The bone may be demineralized bone or non-demineralized bone. “Demineralized bone matrix (DBM)” as used herein refers to bone having less than about 8 wt % residual calcium. Demineralization involves treating a bone tissue to remove its inorganic mineral hydroxyapatite material. The level of demineralization of a bone tissue is defined by the amount (wt %) of residual calcium found in the demineralized bone. In some embodiments, the demineralized bone may still contain physiologically active levels of growth and differentiation factors (e.g., osteogenic growth factors, such as bone morphogenetic proteins (BMPs)) remaining from the initial bone even after the demineralization treatment. In further embodiments, the demineralized bone may contain collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, and mixtures thereof. “Non-demineralized bone” as used in the present application refers to bone that has not been treated to remove minerals present such as, for example, hydroxyapatite.

[0025] The extracellular matrix of bone is comprised primarily of collagen type I and inorganic bone mineral hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). “Collagen type I” refers to a heterotrimer of two $\alpha 1(\text{I})$ and one $\alpha 2(\text{I})$ chains, which is the most common form of fibrillar collagen in a body. Collagen type I is present in the form of elongated fibers greater than 500 μm in length and 500 nm in diameter. These building blocks of the collagen are rod-like triple helices that are stabilized by intramolecular hydrogen bonds between Gly and Hyp in adjacent chains. Dissolving the collagen type I in a solvent and maintaining its native biological activities, however, has been challenging because its native conformation can be degraded by a disruption in the intermolecular crosslinks of the aldimine or keto-imine type and/or a disruption in the intramolecular bonds of the triple helical structure. For example, dilute acidic solvents have been used to dissolve collagen by breaking the intermolecular crosslinks of its aldimine type, while proteolytic enzymes, such as pepsin, have been used to cleave the more stable crosslinks of its keto-imine

type. Moreover, dissolving and electro-spinning collagen in fluoroalcohol solvents, such as 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFP) and 2, 2, 2-trifluoroethanol (TFE), have been shown to result in the significant disruption in the intramolecular bonds of the collagen triple helices (Zeugolis et al., *Biomaterials* 29, 2008, 2293-2305). For the same reasons, it has been challenging to dissolve the extracellular matrix of bone comprised primarily of collagen type I in a solvent while maintaining its native biological activities.

[0026] Moreover, the extracellular matrix of bone is comprised of a complex mixture of the collagen type I, the inorganic bone mineral hydroxyapatite, other proteins (e.g. laminins, fibronectin, elastin, and growth factors), other minerals (e.g. calcium phosphate, magnesium, carbonate, and fluoride ions), and carbohydrates (e.g. parts of glycoproteins). Accordingly, dissolving bone has been even more challenging in view of the complex mixture of the various components in the bone.

[0027] Fluoroalcohol solvents (e.g. HFP and TFE), have been shown to disrupt the intramolecular bonds of the collagen triple helices and thus were not considered as a desirable candidate for dissolving collagen type I. The present invention surprisingly shows that dissolving bone in an anhydrous solvent comprising a highly volatile fluoroalcohols (e.g. HFP, TFE, and their variants) and trifluoroacetic acid (TFA), would still maintain the native structure and/or triple collagen structure of the collagen type I in the bone. In some embodiments, collagen type I in the bone matrix solution prepared according to the methods described herein consists of collagen in its native structure or in its native triple helical structure. In other embodiments, collagen type I in the bone matrix solution prepared according to the methods described herein essentially consists of collagen in its native structure or its native triple helical structure. In additional embodiments, more than about 99 %, 98 %, 97 %, 96 %, 95 %, 90 %, 80 %, 70 %, 60 %, 50 %, 40 %, 30 %, 20 %, 10 %, 5 %, 3% or 1% of collagen type I in the bone matrix solution prepared according to the methods described herein is collagen in its native structure or in its native triple helical structure. In yet additional embodiments, less than about 100%, 99 %, 98 %, 97 %, 96 %, 95 %, 90 %, 80 %, 70 %, 60 %, 50 %, 40 %, 30 %, 20 %, 10 %, 5 %, or 3% collagen type I in the bone matrix solution prepared according to the methods described herein is collagen in its native structure. In further embodiments, between about 100% and about 95%, between about 100% and about 85%, between about 95% and about 85%, between about 85% and about 75%, between about 85% and about 65%, between about 75% and about 65%, between about 65% and about 45%, between about 65% and about 55%, between about 55% and about 35%, between about 55% and about 45%, between about 45% and about 25%, between about 45% and about 35%, between about 35% and about 15%, between about 35% and about 25%, between about 25% and about 5%, or between about 25% and about 15% of collagen type I in

the bone matrix solution prepared according to the methods described herein is collagen in its native structure or in its native triple helical structure.

[0028] The solvents of the present invention may be anhydrous solvents or non-anhydrous solvents with a protectant. The protectant prevents heat or solvent related degradation or denaturation of proteins in the non-anhydrous solvents. The protectants include, but are not limited to, bovine serum albumin and human serum albumin, which has been shown to enhance the long term stability of proteins dissolved in organic solvents.

[0029] Anhydrous trifluoroacetic acid (TFA) has been used in the peptide synthesis to cleave an N-terminal amino acid of a purified synthetic peptide, such as polypeptide models of collagen (Guantieri and Tamburro, *Int. J. Peptide Protein Res.*, 2, 1984, 274-278; Fields et al., *Biopolymers*, 33, 11, 1993, 1695-1707). The extracellular matrix of bone, however, is comprised of a complex mixture of carbohydrates, proteins, and minerals and thus is difficult to be dissolved in the same solution as the purified synthetic peptide. For example, as shown in the comparative examples herein, a solvent having 50% TFA and 50% dimethylformamide (DMF) was not able to dissolve the bone even after an extended period of stirring on the order of 14-28 days at 4°C or 22°C. The present invention surprisingly shows an anhydrous solvent comprising TFA and a volatile fluoroalcohol (e.g. HFP, TFE, and their variants) effectively dissolves bone having complex mixtures of carbohydrates, proteins, and minerals. In some embodiments of the present invention, a secondary solvent may be added to the TFA and fluoroalcohol solvent. Specifically, the secondary solvent may be selected from the group consisting of the family of hexa-fluoro-isopropanol (HFP), N-methyl morpholine N-oxide, calcium nitrate-methanol, or related organic solvents.

[0030] The anhydrous solvent to dissolve the bone according to some embodiments of the present invention may comprise (i) hexafluoroisopropanol (HFP), 2, 2, 2-trifluoroethanol (TFE) or their variants, and (ii) trifluoroacetic acid (TFA). As used herein, the variants of HFP and TFE refers to other fluoroalcohol solvents, including, but not limited to, 2,3-difluorobenzyl alcohol, 1,3-difluoro-2-propanol, trifluoroethanol, 2,2,3,3,4,4,4-heptafluoro-1-butanol, 3,3,4,4,5,5,5-heptafluoro-2-pentanol, 2,2,3,3,4,4,5,5-octafluoro-1-pentanol, 1H,1H,2H,2H-perfluoro-1-decanol, 1H,1H,2H,3H,3H-perfluorononane-1,2-diol, 1H,1H,9H-perfluoro-1-nonanol, 1H,1H-perfluorooctanol, 1H,1H,2H,2H-perfluorooctanol, 1H, 1H, 2H, 3H, 3H-perfluoroundecane-1,2-diol, trifluorobutanol, 1,1,1-trifluoro-2-propanol, 3,3,3-trifluoro-1-propanol, 1,1,1,3,3,3-hexafluoro-2-propanol, 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol, 2,2,3,4,4,4-hexafluoro-1-butanol, 2-trifluoromethyl-2-propanol, 2,2,3,3-tetrafluoro-1-propanol, 3,3,3-trifluoro-1-propanol, 4,4,4-trifluoro-1-butanol, and a mixture thereof. In further embodiments, the anhydrous solvent to dissolve the bone may consist of (i) HFP, TFE or their variants, and (ii) trifluoroacetic acid (TFA). In

additional embodiments, the anhydrous solvent to dissolve the bone may exclude dimethylformamide (DMF).

[0031] In one aspect, the amount of HFP, TFE, their variants in the solvent may be about 95 wt%, 90 wt%, 80 wt%, 70 wt%, 60 wt%, 50 wt%, 40 wt%, 30 wt%, 20 wt%, 10 wt%, 5 wt%, between about 95 wt% and about 5 wt%, between about 95 wt% and about 10 wt%, between about 95 wt% and about 25 wt%, between about 95 wt% and about 45 wt%, between about 95 wt% and about 65 wt%, between about 95 wt% and about 85 wt%, between about 95 wt% and about 90 wt%, between about 90 wt% and about 5 wt%, between about 90 wt% and about 10 wt%, between about 90 wt% and about 25 wt%, between about 90 wt% and about 45 wt%, between about 90 wt% and about 65 wt%, between about 90 wt% and about 85 wt%, between about 80 wt% and about 5 wt%, between about 80 wt% and about 10 wt%, between about 80 wt% and about 25 wt%, between about 80 wt% and about 45 wt%, between about 80 wt% and about 65 wt%, and between about 80 wt% and about 75 wt%. In another aspect, the amount of TFA in the solvent may be at least about 1, 2, 3, 4, or 5 wt%, 6 wt%, 7 wt%, 8 wt%, 9 wt%, 10 wt%, 13 wt%, 15 wt%, 20 wt%, 25 wt%, 30 wt%, 40 wt%, 50 wt%, 60 wt%, 70%, 80 wt%, or 90 wt% between about 95 wt% and about 5 wt%, between about 95 wt% and about 10 wt%, between about 95 wt% and about 25 wt%, between about 95 wt% and about 45 wt%, between about 95 wt% and about 65 wt%, between about 95 wt% and about 85 wt%, between about 95 wt% and about 90 wt%, between about 90 wt% and about 5 wt%, between about 90 wt% and about 10 wt%, between about 90 wt% and about 25 wt%, between about 90 wt% and about 45 wt%, between about 90 wt% and about 65 wt%, between about 90 wt% and about 85 wt%, between about 80 wt% and about 5 wt%, between about 80 wt% and about 10 wt%, between about 80 wt% and about 25 wt%, between about 80 wt% and about 45 wt%, between about 80 wt% and about 65 wt%, and between about 80 wt% and about 75 wt%.

[0032] As used herein, the term “about” modifying, for example, the quantity of an ingredient in a composition, concentrations, volumes, process temperature, process time, yields, flow rates, pressures, and like values, and ranges thereof, refers to variation in the numerical quantity that can occur, for example, through typical measuring and handling procedures used for making compounds, compositions, concentrates or use formulations; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of starting materials or ingredients used to carry out the methods; and like considerations. The term “about” also encompasses amounts that differ due to aging of, for example, a composition, formulation, or cell culture with a particular initial concentration or mixture, and amounts that differ due to mixing or processing a composition or formulation with a particular initial concentration or mixture. Whether modified by the term “about” the claims appended hereto include equivalents to

these quantities. The term “about” further may refer to a range of values that are similar to the stated reference value. In certain embodiments, the term “about” refers to a range of values that fall within 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 percent or less of the stated reference value.

[0033] The amount of the anhydrous solvent used in preparing the bone matrix solution according to some embodiments of the present invention is the amount sufficient to dissolve the bone and thus sufficient to form the bone matrix solution at a temperature described as follows. In some aspects, the bone may be dissolved in the solvent described herein at about 37 °C, 36 °C, 35 °C, 34 °C, 33 °C, 32 °C, 31 °C, 30 °C, 29 °C, 28 °C, 27 °C, 26 °C, 25 °C, 24 °C, 23 °C, 22 °C, 21 °C, 20 °C, 19 °C, 18 °C, 17 °C, 16 °C, 15 °C, 14 °C, 13 °C, 12 °C, 11 °C, 10 °C, 9 °C, 8 °C, 7 °C, 6 °C, 5 °C, 4 °C or lower. In other aspects, the bone may be dissolved in the solvent described herein at a temperature lower than about 37 °C, 36 °C, 35 °C, 34 °C, 33 °C, 32 °C, 31 °C, 30 °C, 29 °C, 28 °C, 27 °C, 26 °C, 25 °C, 24 °C, 23 °C, 22 °C, 21 °C, 20 °C, 19 °C, 18 °C, 17 °C, 16 °C, 15 °C, 14 °C, 13 °C, 12 °C, 11 °C, 10 °C, 9 °C, 8 °C, 7 °C, 6 °C, 5 °C, or 4 °C. In further aspects, the bone is dissolved in the solvent described herein at a temperature high enough to effectively break the bonds in a solid. In some embodiments, the bone may be dissolved in the solvent described herein at a temperature higher than about 20 °C, 19 °C, 18 °C, 17 °C, 16 °C, 15 °C, 14 °C, 13 °C, 12 °C, 11 °C, 10 °C, 9 °C, 8 °C, 7 °C, 6 °C, 5 °C, 4 °C, 3 °C, 2 °C, 1 °C, or 0 °C. It has been found by the inventors that the temperature in which the bone is dissolved has an effect on the native structure and/or the native triple helical structure of collagen in the bone. In some embodiments, dissolving bone at temperatures below about room temperature, which is defined as 25 °C, has provided an improved collagen product in its native structure and/or in its native triple helical structure compared to dissolving bone at higher temperatures. In some embodiments, bone is dissolved at a temperature below 22 °C. In other embodiments, bone is dissolved at a temperature between about 4 °C and about 21 °C.

[0034] The amount of the bone dissolved in the solvent according to some embodiments of the present invention may be between about 0.001 mg/mL and about 300 mg/mL, between about 1 mg/mL and about 300 mg/mL, between about 10 mg/mL and about 300 mg/mL, between about 20 mg/mL and about 300 mg/mL, between about 0.001 mg/mL and about 200 mg/mL, between about 1 mg/mL and about 320 mg/mL, between about 10 mg/mL and about 200 mg/mL, between about 20 mg/mL and about 200 mg/mL, between about 30 mg/mL and about 150 mg/mL, between about 50 mg/mL and about 150 mg/mL, between about 100 mg/mL and about 150 mg/mL, between about 130 mg/mL and about 150 mg/mL, between about 30 mg/mL and about 100 mg/mL, or between about 30 mg/mL and about 50 mg/mL. According to additional embodiments of the present invention, the bone may be dissolved by

stirring the bone in the solvent described herein for more than about 3 hours, 6 hours, 12 hours, 24 hours, 36 hours, 48 hours, 3 days, 4 days, 5 days or 6 days. In another aspect, the bone may be dissolved by stirring the bone in the solvent described herein for less than about 5 hours, 10 hours, 15, hours, 20 hours, 25 hours, 30 hours, 40 hours, 50 hours, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In another aspect, the bone may be dissolved by stirring the bone in the solvent described herein for about 3 hours, 5 hours, 6 hours, 10 hours, 12 hours, 15, hours, 20 hours, 24 hours, 25 hours, 30 hours, 40 hours, 50 hours, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 20 days, 30 days, 40 days, or 50 days. According to yet additional embodiments of the present invention, the bone to be dissolved in the solvent described herein may be in the forms of particles and/or powder. Demineralized bone particles may be prepared from cleaned and disinfected bone fragments that have been freeze-dried and ground/fractured into bone particles. Bone particles may be selected by, for example, using sieving devices (i.e., mesh sieves) commercially available to obtain particles within a desired size range. Such demineralized bone particles may have an average diameter of between about 125 microns and about 4 mm; between about 710 microns and about 2 mm; between about 125 microns and about 500 microns; between about 125 microns and about 850 microns; or between about 250 microns and about 710 microns. Certain embodiments of the present invention may include demineralized bone powder that is commercially available. For example, a suitable demineralized bone powder that is widely and reliably available is produced by LifeNet Health, Virginia Beach, VA.

[0035] The invention also relates to methods of preparing a bone matrix implant with a predetermined shape. An “implant” refers to any object that is designed to be placed partially or wholly within a patient's body for one or more therapeutic or prophylactic purposes such as for tissue augmentation, contouring, restoring physiological function, repairing or restoring tissues damaged by disease or trauma, and/or delivering therapeutic agents to normal, damaged or diseased organs and tissues. A “bone matrix implant” refers to a medical device or implant that includes a volume replacement material for augmentation or reconstruction to replace a whole or part of a bone structure. A predetermined shape of the bone matrix implant may be varied to fit the implant site. A “bone matrix” refers to any mixture of the volume replacement materials for augmentation or reconstruction to replace a whole or part of a bone structure.

[0036] The methods of preparing a bone matrix implant with a predetermined shape according to some embodiments of the present invention comprises dissolving a bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP), 2, 2, 2-trifluoroethanol (TFE) or isopropanol and (ii) at least

about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution; exposing the bone matrix solution to conditions sufficient to evaporate an amount of the solvent to form a colloidal gel; and allowing the colloidal gel to harden into the predetermined shape to form the bone matrix implant. In some embodiments, the colloidal gel may be placed in a mold to form the predetermined shape, formed with or without particles of bone in the suspension. As used herein, a “colloidal gel” refers to a colloid that has developed resistance to flow and thus is in a more solid form than a solution. A “colloid” refers to a system composed of a continuous medium throughout which are distributed small particles, e.g., 1 to 1000 nm in size or larger, that do not settle out under the influence of gravity; the particles may be in emulsion or in suspension. The invention further relates to a bone matrix implant produced by the method described herein.

[0037] Methods to evaporate the solvent to form a colloidal gel from the bone matrix solution according to some embodiments of the present invention may include, but are not limited to, air drying, vacuum drying, heat drying, and freeze drying. The amount of the solvent to form a colloidal gel from the bone matrix solution according to some embodiments of the present invention may be about 98 wt%, 95 wt%, 90 wt%, 85 wt%, 80 wt%, 75 wt%, 70 wt%, 65 wt%, 60 wt%, 55 wt%, 50 wt%, 45 wt%, 40 wt%, 35 wt%, 30 wt%, 25 wt%, 20 wt%, 15 wt%, 10 wt%, 5 wt%, or 0 wt%. In one aspect, the amount of the solvent to form a colloidal gel from the bone matrix solution may be less than about 98 wt%, 95 wt%, 90 wt%, 85 wt%, 80 wt%, 75 wt%, 70 wt%, 65 wt%, 60 wt%, 55 wt%, 50 wt%, 45 wt%, 40 wt%, 35 wt%, 30 wt%, 25 wt%, 20 wt%, 15 wt%, 10 wt%, or 5 wt%. In another aspect, the amount of the solvent to form a colloidal gel from the bone matrix solution may be more than about 98 wt%, 95 wt%, 90 wt%, 85 wt%, 80 wt%, 75 wt%, 70 wt%, 65 wt%, 60 wt%, 55 wt%, 50 wt%, 45 wt%, 40 wt%, 35 wt%, 30 wt%, 25 wt%, 20 wt%, 15 wt%, 10 wt%, 5 wt%, or 0 wt%.

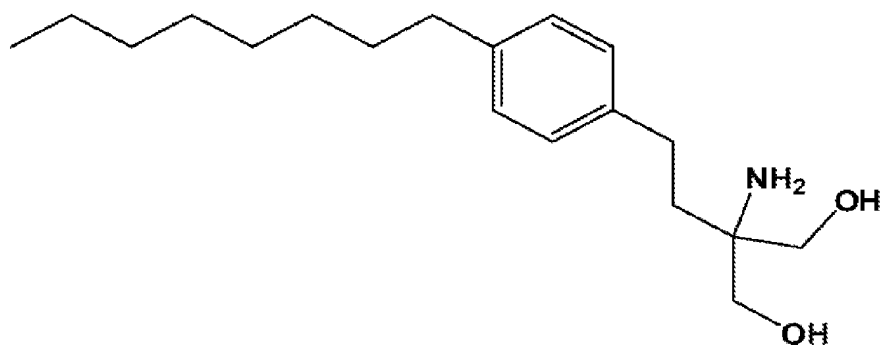
[0038] The methods of preparing a bone matrix implant with a predetermined shape according to some embodiments of the present invention may further comprise adding a bioactive factor to the bone matrix solution described herein. A “bioactive factor” refers to protein, carbohydrate, or mineral that has any effect on a cellular activity. Examples of bioactive factors include, but are not limited to, an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor (TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein, erythropoietin, thrombopoietin,

tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), or an interleukin, among others. The bioactive factor may be a BMP, PDGF, FGF, VEGF, TGF, insulin, among others. Examples of BMPs include but are not limited to BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and a mixture thereof.

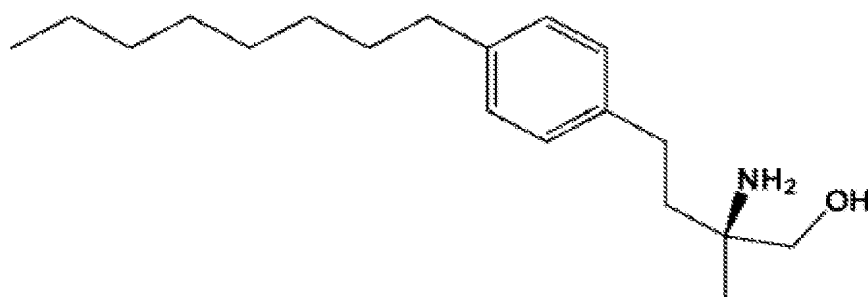
[0039] In some embodiments, the bioactive factor may include chemokines. Chemokines refers to a family of small proteins secreted from cells that promote the movement or chemotaxis of nearby cells. Some chemokines are considered pro-inflammatory and can be induced during an immune response while others are considered homeostatic. Typically, chemokines exert their chemoattractant function and other functions by binding to one or more chemokine receptors. Chemokines include proteins isolated from natural sources as well as those made synthetically, by recombinant means or by chemical synthesis. Exemplary chemokines include, but are not limited to, MCP-1, Eotaxin, SDF-1 β , GRO- α , MIP-1 β , IL-8, IP-10, MCP-3, MIP-3 α , MDC, MIP-1 α , BCA-1, GCP-2, ENA-78, PBP, MIG, PF-4, PF-4-var1, SDF-2, MCP-2, MCP-4, MIP-4, MIP-3 β , MIP-2 α , MIP-2 β , MIP-5, HCC-1, RANTES, Eotaxin-2, TARC, I-309, Lymphotactin, Lungkine, C10, MIP-1 γ , MCP-5, LEC, Exodus-2, MIP-3, TECK, Eotaxin-3, CTACK, MEC, SCM-1 β , I-TAC, BRAK, SR-PSOX, Fractalkine, LD78- β , MIP-1b2, and others known to those of skill in the art. References to chemokines typically include monomeric forms of such chemokines. Chemokines also include dimeric or other multimeric forms.

[0040] In additional embodiments, the bioactive factor may also include small molecule drugs. Small molecule drugs include a molecule, whether naturally-occurring or artificially created (e.g., via chemical synthesis) that has a relatively low molecular weight and that is not a protein, a nucleic acid, or a carbohydrate. In one aspect, though not necessarily, small molecule drugs are monomeric and have a molecular weight of less than about 1500 g/mol. In another aspect, the drug is one that has already been deemed safe and effective for use by the appropriate governmental agency or body. For example, drugs for human use listed by the FDA under 21 C.F.R. §§ 330.5, 331 through 361 and 440 through 460; drugs for veterinary use listed by the FDA under 21 C.F.R. §§ 500 through 589, incorporated herein by reference, are all considered acceptable for use in inventive conjugates. In another aspect, the small molecule drugs may include agonists of a Sphingosine-1 -phosphate (SIP) agonist, such as fingolimod (FTY720), which is a synthetic compound that acts as an agonist of the S1P1, S1P3, S1P4, and S1P5 receptors when phosphorylated into FTY720P. For example, the small molecule drugs may include the following molecules:

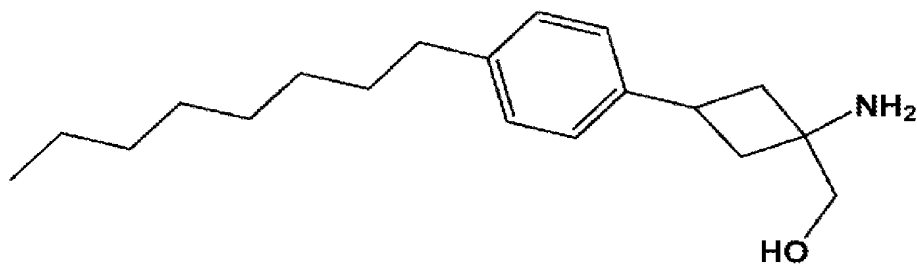
FTY720 (fingolimod)



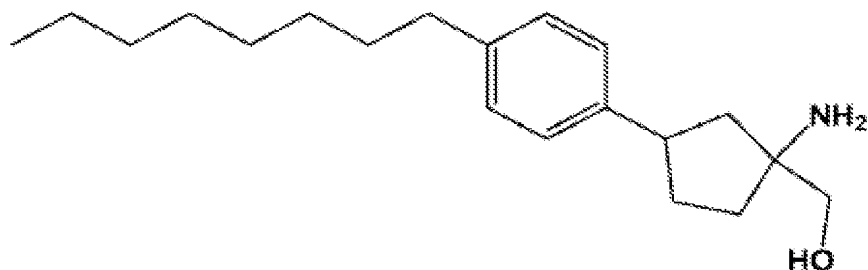
2-amino-2-(4-octylphenethyl)propane-1,3-diol,



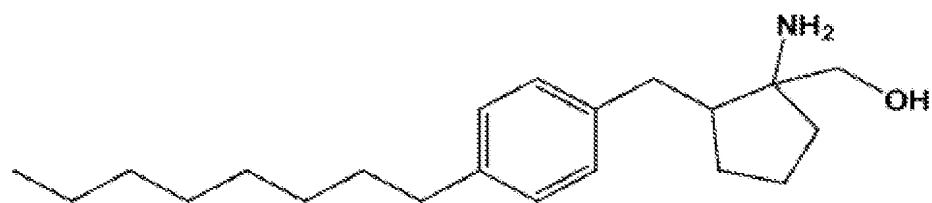
(5')-2-amino-2-methyl-4-(4-octylphenyl)butan-1-ol,



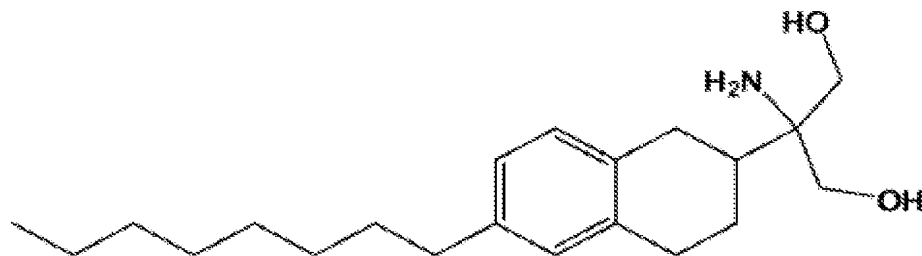
(1-amino-3-(4-octylphenyl)cyclobutyl)methanol,



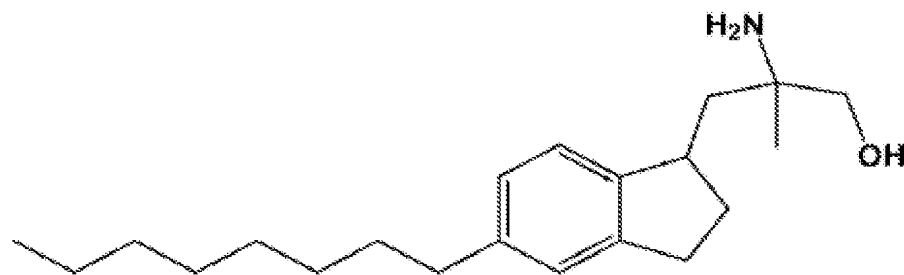
(1-amino-3-(4-octylphenyl)cyclopentyl)methanol,



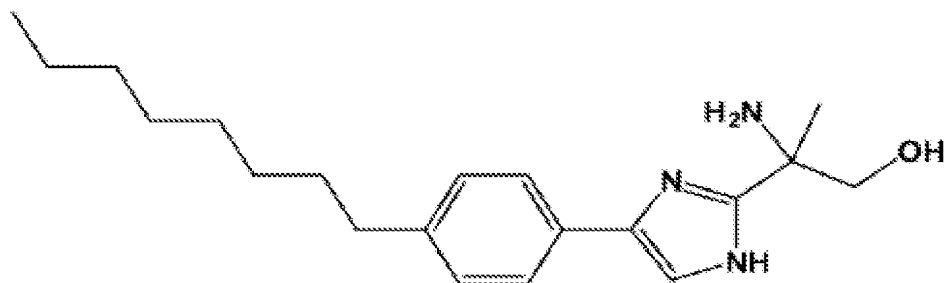
(1 -amino-2-(4-octylbenzyl)cyclopentyl)methanol,



2-amino-2-(6-octyl-1,2,3,4-tetrahydronaphthalen-2-yl)propane-1,3-diol,



2-amino-2-methyl-3-(5-octyl-2,3-dihydro-1H-inden-1-yl)propan-1-ol, and



2-amino-2-(4-(4-octylphenyl)-1H-imidazol-2-yl)propan-1-ol.

[0041] The bone matrix implants may further comprise a tissue, an organ, a biocompatible matrix and/or a mixture of two or more thereof. The bone matrix implants may comprise bone, cartilage, and/or connective tissue.

[0042] The invention also relates to methods of preparing a film of bone matrix on a surface. The method of preparing a film of bone matrix on a surface according to some embodiments of the present invention comprises dissolving a bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP), 2, 2, 2-trifluoroethanol (TFE) or isopropanol, and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution; exposing the bone matrix solution to conditions sufficient to evaporate an amount of the solvent to form a colloidal gel; coating the surface with the colloidal gel, and exposing the coated surface to conditions sufficient to further evaporate an amount of solvent to form a film. The invention further relates to a synthetic surface coated with the bone matrix produced by the method described herein.

[0043] In some embodiments, the film of the bone matrix may be formed on a surface of another biocompatible matrix. In further embodiments, the biocompatible matrix is an implantable biocompatible matrix that can function as a support system for the bone matrix described herein. A biocompatible matrix should be non-toxic, non-eliciting or stimulating severe inflammatory response or immunological rejections, and devoid of other undesired reactions at the implantation site. In one embodiment, the biocompatible matrix is another bone matrix or cartilage or connective tissue.

[0044] In some embodiments, the film of the bone matrix may be formed on a surface of biocompatible matrix including, but not limited to, bone graft implants, synthetic bone graft materials in forms of particulates, sheet, or blocks, tendon and/or ligament in bone tunnels, prosthetic implant, for example, for hip, shoulder, knee, or ankle, and trabecular metal. In further embodiments, the bone graft implants may include allograft or xeno-graft. In yet further embodiments, the bone graft implants may include structural bone implants including, but not limited to, monolithic or composite spinal implants (e.g., VERTIGRATs from LifeNet Health) and bone struts or blocks. In yet further embodiments, the bone graft implants may include demineralized, or non-demineralized bone particulates, including, but not limited to, cortical, cancellous, or cortical cancellous bone. In other embodiments, the film of the bone matrix may be formed on cell or tissue culture surface.

[0045] Suitable biocompatible matrices include, but are not limited to, porous biocompatible scaffolds into which bone cells or progenitor cells may migrate. Osteogenic or chondrogenic cells, i.e., cells involved in the process of deposition of new bone material or cartilaginous material, respectively, can often attach to such porous biocompatible matrices, which can then serve as scaffolding for bone and cartilage tissue growth. Cells involved in the process of deposition of new ligament or tendon material can also attach to such porous biocompatible matrices. For certain applications, the biocompatible matrix should have sufficient mechanical strength to maintain its three dimensional structure and help support

the immobilization of the bone segments being united or grafted together. Porous biocompatible matrices which provide scaffolding for tissue growth can accelerate the deposition of new bone or the rate of bone growth and are said to be “osteoconductive.” Osteoconductive biocompatible matrices are especially useful in the pharmaceutical compositions described herein. Porous biocompatible matrices which provide scaffolding for tissue growth can accelerate the deposition of new cartilage or the rate of cartilage growth and are said to be “chondroconductive.” Osteoconductive biocompatible matrices are especially useful in the pharmaceutical compositions described herein. Chondroconductive biocompatible matrices are especially useful in the pharmaceutical compositions described herein. The osteoinductive or chondroinductive activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating. Thus, the osteoconductive or chondroconductive activity of the biocompatible matrices treated with the bone matrix of the present invention may be enhanced compared to matrices not treated with the bone matrix described herein. Of course, the biocompatible matrices are considered to be osteoconductive or chondroconductive if cells within the biocompatible matrix begin to differentiate into more osteoblast-like or chondrocyte-like appearing or functional cells, respectively.

[0046] The biocompatible matrices according to some embodiments of the present invention can be derived from natural sources or they can be synthetic or a mixture of both. Biocompatible matrices from natural sources may also comprise natural polymers, including, but not limited to, collagen, hyaluronic acid, alginate, albumin, fibrinogen-fibrin, chitosan, elasin, laminin, connective tissues, intervertebral disc, cortical or cancellous bone, demineralized or mineralized bone, fascia lata, dermis, muscle, ligament, tendon, cartilage including articular/hyaline cartilage, elastic cartilage, and fibrocartilage, a mixture thereof, and mixture of reconstituted tissue. Biocompatible matrices from synthetic sources refer to any material not produced by living organisms, which may include, not limited to, the synthetic material made up of organic components, inorganic components, or a mixture thereof. In some embodiments, a synthetic biocompatible matrix may comprise an organic synthetic polymer, such as poly(lactic-co-glycolic acid), polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA), polyhydroxybutyrate (PHB), Poly(ethylene glycol) (PEG), poly(ethylene oxide) (PEO)), and others. In some embodiments, a tissue, an organ, or biocompatible matrix comprising at least one of alginate, chitosan, collagen, gelatin, hyaluronic acid, a fibronectin, an elastin, a laminin, and a proteoglycan may be employed. In certain embodiments, a biocompatible matrix comprising inorganic components, such as hydroxyapatite, calcium sulfate, octacalcium phosphate, calcium phosphate, macroporous calcium metaphosphate ceramic, β -tricalcium phosphate, metal, metal alloy, and others, may be used. A biocompatible matrix used in certain embodiments of the present invention may be prepared by

demineralizing, decellularizing or devitalizing a tissue or an organ and cells may be seeded onto the biocompatible matrix.

[0047] In some embodiments, the bone matrix described herein may be applied to another biocompatible matrix and may be incubated at conditions permitting the generation of a matrix that is partially or fully coated with the bone matrix. In some embodiments, incubation may be carried out at about 40 °C or lower, or between about 10 °C and about 37 °C, or about 20 °C and about 37 °C. Incubation may be carried out for between at least about 2 minutes and about 120 minutes, about 3 minutes and about 100 minutes, about 4 minutes and about 80 minutes, about 5 minutes and about 60 minutes, and about 5 minutes and about 30 minutes in certain embodiments. Incubation may be performed under static or dynamic conditions, such as with agitation, shaking, stirring, mixing, horizontal motion, rocking, and others.

[0048] In some embodiments of the present invention, a biocompatible matrix may be lyophilized before the bone matrix is applied to the biocompatible matrix. In certain embodiments, the bone matrix may be coated on the biocompatible matrix, and the coated matrix may be subsequently lyophilized. The lyophilized, coated matrix can then be rehydrated before it is used. Further, the cells can be seeded onto the matrix before implantation.

[0049] Examples of suitable osteoconductive or chondroconductive biocompatible matrices include but are not limited to, collagen (e.g., bovine dermal collagen), fibrin, calcium phosphate ceramics (e.g., hydroxyapatite and tricalcium phosphate), calcium sulfate, guanidine-extracted allogenic bone and combinations thereof. A number of suitable biocompatible matrices are commercially available, such as CollograftTM (Collagen Corporation), which is a mixture of hydroxyapatite, tricalcium phosphate and fibrillar collagen, and InterporeTM (Interpore International), which is a hydroxyapatite biomatrix formed by the conversion of marine coral calcium carbonate to crystalline hydroxyapatite.

[0050] A number of synthetic biodegradable polymers can serve as osteoconductive or chondroconductive biocompatible matrices with sustained release characteristics. Descriptions of these polymers can be found in Behravesch (1999) Clinical Orthopaedics 367, S118 and Lu (2000) Polymeric Delivery Vehicles for Bone Growth Factors in Controlled Drug Delivery: Designing Technologies for the Future, Park and Mrsny eds., American Chemical Society, which is incorporated herein in its entirety. Examples of these polymers include poly α -hydroxy esters such as polylactic acid/polyglycolic acid homopolymers and copolymers, polyphosphazenes (PPHOS), polyanhydrides and poly(propylene fumarates).

[0051] Polylactic acid/polyglycolic acid (PLGA) homo and copolymers are well known in the art as sustained release vehicles. The rate of release can be adjusted by the skilled artisan by variation of polylactic acid to polyglycolic acid ratio and the molecular weight of the polymer (see Anderson (1997) *Adv. Drug Deliv. Rev.* 28:5. The incorporation of PEG into the polymer as a blend to form microparticle matrices allows further alteration of the release profile of the active ingredient (see Cleek (1997) *J. Control Release* 48, 259). Ceramics such as calcium phosphate and hydroxyapatite can also be incorporated into the formulation to improve mechanical qualities.

[0052] In one embodiment, the biocompatible matrices used in the methods of the present invention are other types of bone matrices. For example, the bone matrix prepared by the methods described herein can be coated on another type of bone matrix. As used herein, the other types of the bone matrix may be a biocompatible matrix derived from or including elements of natural bone. In some embodiments, the natural bone is mineralized, partially demineralized, demineralized, cancellous, cortical, or cortical cancellous bone. The bone matrices used herein may or may not include additional synthetic components not typically found in bone tissue. Other embodiments include methods utilizing a biocompatible matrix derived from cartilage, other soft tissues such as the dermis, connective tissue, fascia, small intestine submucosa, serous membrane, pericardium, tendon, ligament, muscle, adipose tissue, myelin, blood vessels, base membrane, amniotic membrane and others. A biocompatible matrix prepared from hyaline cartilage, fibrocartilage or elastic cartilage, may be employed in some embodiments. A biocompatible matrix may be prepared from hyaline cartilage found in the condyle, tibial plateau, femoral head, humeral head, costal cartilage, or fibrocartilage found in intervertebral discs, or elastic cartilage found in the epiglottis or ear. In certain embodiments, a biocompatible matrix derived from natural sources that has been optionally cleaned, disinfected, chemically modified, decellularized, particulated, homogenized, lyophilized, gamma ray irradiated, and/or plasticized may be used. Any of the biocompatible matrices used herein may or may not include additional synthetic components not typically found in such tissue.

[0053] In one specific embodiment, the bone or cartilage biocompatible matrices may be demineralized or decellularized, respectively. Examples of demineralized matrices and methods of making are described in U.S. Patent No. 6,189,537 and 6,305,379, which are incorporated herein in its entirety.

[0054] The biocompatible matrix, tissue, or organ used in certain embodiments of the present invention may be in the form of a powder, particulates, sheets, fibers, gels, putties, paste, blocks, cylinders, sponges, meshes, films, slices, curls, flakes, or wedges, among others. In certain embodiments of the present invention, the biocompatible matrix, tissue, or organ treated with the bone matrix by the methods described herein may be in the form of a powder, fibers, putty, or a sponge. In further embodiments, the

sponge can include, for example, the implant having sponge-like structures disclosed in the co-pending, commonly-assigned patent application PCT/US09/04556 entitled “Composition for a Tissue Repair Implant and Methods of Making the Same” filed on August 7, 2009, which is incorporated herein in its entirety. The treated matrices can be used in any of the methods of the present invention.

[0055] The invention also relates to methods of cell culture comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, cell culture refers to the maintenance of cells in an artificial environment, commonly referred to as an *in vitro* environment. The term cell culture is a generic term and may be used to encompass the cultivation not only of individual cells, but also of tissues, organs, organ systems or whole organisms. The cells used in the culture methods disclosed herein can be any prokaryotic or eukaryotic cell. The cell type used in the culture methods disclosed herein need not be from the same species from which the cell support compositions derive. In addition, the cells may be from an established cell line, or they may be primary cells or genetically engineered cells.

[0056] For example, the invention provides for growing and/or culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. “Growing and/or culturing cells on a cell culture surface” includes traditional cell culture methods as well as placing on a surface in any setting, such as in natural or synthetic biocompatible matrices or tissues. The cells may be mammalian, such as but not limited to human, bovine, porcine, murine, ovine, equine, canine, feline and others. In some embodiments, the cells that are cultured on the substrate coated with the bone matrix are stem cells. As used herein, a stem cell is used as it is in the art and means a cell that has the ability to divide and give rise to one daughter cell that may be at least partially differentiated and to another daughter cell that retains the developmental potential of the mother cell. As used herein, stem cells can be adipose derived stem cells, dental pulp stem cells, adult stem cells (ASCs), embryonic stem cells (ESCs), committed progenitor cells, and/or induced pluripotent stem cells (iPSCs). In further embodiments, the bone matrix coated surface can be used in *in vitro* methods for supporting cell growth and proliferation as well as for increasing osteogenesis, chondrogenesis, or ligament/tendon genesis in the stem cells cultured on the bone matrix coated surface.

[0057] In some embodiments, the cells may be mesenchymal stem cells, such as adipose-derived stem cells, embryonic stem cells, progenitor cells, differentiated cells, undifferentiated cells, and/or -pluripotent stem cells. Appropriate cells may also include, but are not limited to cells of the ectodermal lineage, cells of the mesodermal lineage, and cells of the endodermal lineage. Examples of cells of the ectodermal lineage include but are not limited to keratinocytes, osteoblasts, chondrocytes, neurons. Examples of cells

of the mesodermal lineage include but are not limited to myoblasts, adipocytes, fibroblasts, endothelial cells, or stromal cells. Examples of cells of the endodermal lineage include but not limited to epithelial cells of the auditory tube, the respiratory tract, such as trachea, bronchi, and alveoli of the lungs, the gastrointestinal tract, the urinary bladder and epithelial cells lining all glands. The cells may also be primary cells derived from tissues or organs. Appropriate cell lines used in the present invention may include but are not limited to mesenchymal cell lines, preosteoblastic cell lines, osteoblastic cell lines, and chondroblastic cell lines. The bone matrix may be coated directly into a tissue, organism or other setting such as a matrix, including, but not limited to, bone matrices.

[0058] In some embodiments, the cells may be derived from autologous or allogeneic sources. The cells may be differentiated cells including chondrocytes, osteoblasts, osteoclasts, endothelial cells, epithelial cells, fibroblasts, and periosteal cells. Additionally, the cells may be totipotent, pluripotent, multipotent, progenitor, or adult somatic stem cells. The stem cells may be derived from embryos, placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, muscle. The stem cells may be derived from skeletal muscle, smooth muscle, and cardiac muscle. The stem cells may be derived from genetic reprogramming of mature cells, such as induced pluripotent stem cells (iPSCs). All cells may further be derived from living or recently deceased donors.

[0059] Any cell described herewith may be cultured on a cell culture surface coated with the bone matrix produced by the method described herein for between about 15 minutes and about 4 weeks, about 2 hours and about 2 weeks, about 2 hours and about 1 week, about 2 hours and about 72 hours, about 24 hours and about 72 hours, or about 24 hours and about 96 hours, at between about 20 °C and about 40 °C or about 30°C and about 37°C, in an atmosphere containing between about 1% CO₂ and about 10% CO₂ or about 4% CO₂ and about 6% CO₂, in certain embodiments. In some embodiments of the present invention, cells may be cultured in the presence of one or more modified growth factors and (1) a tissue or an organ, (2) a matrix, or (3) a combination thereof. Cells that have been cultured in the presence of one or more modified growth factors in a cell culture medium may subsequently be applied to a matrix, a tissue, an organ or a combination thereof, in certain embodiments.

[0060] The invention also relates to methods of promoting osteoinductivity, with the methods comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, “osteoinductivity” can refer to causing cells to differentiate into cells that are more osteoblast-like in phenotype, or the term can refer to increasing the proliferation of

osteoblasts, or both. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The osteoinductive activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating.

[0061] The invention also relates to methods of promoting chondroinductivity, with the methods comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, “chondroinductivity” can refer to causing cells to differentiate into cells that are more chondrocyte-like in phenotype, or the term can refer to increasing the proliferation of chondrocytes, or both. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The chondroinductive activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating.

[0062] The invention also relates to methods of promoting ligament/tendon differentiation, with the methods comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, “ligament/tendon differentiation” can refer to causing cells to differentiate into cells that are more ligament and/or tendon-like in phenotype, or the term can refer to increasing the proliferation of ligament and/or tendon, or both. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The cells may be present in culture or in a tissue, organ or portion thereof or in an organism. The ligament/tendon differentiation activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating.

[0063] There are variety of osteoblast, chondrocyte, ligament/tendon differentiation markers that can be measured to assess osteoinductivity, chondroinductivity, or ligament/tendon differentiation, respectively. For example, cells express alkaline phosphatases during the early stages of differentiation toward osteoblast lineages. Therefore, *in vitro* alkaline phosphatase assays may be used to evaluate osteoinductivity in cells cultured on the surface coated by the bone matrix by the methods described herein. The ability of the bone matrix prepared by the methods of the present invention to stimulate or induce the alkaline phosphatase expression in an otherwise non-bone forming cells, such as myoblast (C2C12 cells), would indicate that the bone matrix of the present invention has osteoinductive activity. In

these assays, cells cultured on a surface not coated by the bone matrix of the present invention and on a natural surface without any coating are used as negative controls to show that the baseline alkaline phosphatase expression on non-bone forming cells. The baseline of the osteoblastic markers in the negative control need not be zero, meaning that the cells in the negative control group may have at least some level of phenotypic marker(s). Accordingly, an “osteoinductive” surface of the present invention would simply cause an increase in the osteoblastic markers in experimental cells over control grown on the bone matrix prepared by the methods described herein. Similarly, chondrocyte markers, including but not limited to type X collagen, type II collagen, Sox 9, Aggrecan, Matrilin-1 and CEP-68, to name a few, can be used to assess chondroinductive potential. Moreover, ligament/tendon markers, including but not limited to scleraxis, can be used to assess ligament/tendon differentiation potential.

[0064] Moreover, osteoinductivity, chondroinductivity, and ligament/tendon differentiation may be determined in tissue culture by investigating the ability of the bone matrix prepared by the methods of the present invention to differentiate or induce osteoblast phenotype, chondrocyte phenotype, ligament/tendon cell phenotype in cultured cells, such as primary cells, cell lines, or explants. For example, the cells may display increased production of a marker characteristic of osteoblasts and/or chondrocytes, such as alkaline phosphatase, etc. For example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the surface coated with bone matrix may be more than 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 times greater than those not coated with the bone matrix. In another example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the culture on the bone matrix implant described herein may be more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500 or even 1000 times greater than those of the unmodified growth factors. Of course, this indicates that lower concentrations of modified growth factor, compared to unmodified growth factor are required to achieve the same effects.

[0065] Osteoinductivity, chondroinductivity, ligament/tendon differentiation, for assessing the bone, cartilage, ligament or tendon forming potential induced by the bone matrix of the present invention in a location such as muscle, may also be evaluated using a suitable animal model. For example, intramuscular implantation into a rodent has been used as a model to assess osteoinductive activity of bioactive factors.

[0066] The invention also relates to methods of promoting cell attachment, proliferation or maintaining the differentiated state or preventing de-differentiation of osteoblasts, chondrocytes, ligament cells, tendon cells and/or any cell type disclosed herein with the methods comprising culturing the cells on a cell culture surface coated with the bone matrix produced by the method described herein. The

proliferative activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating.

[0067] Mitogenicity may be assessed by investigating cell proliferation induced by the bone matrix prepared by the methods of the present invention using various in vitro assays that measure metabolic activity, such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, alamarBlue[®] assay, and others. The alamarBlue[®] assay uses a non-cytotoxic reduction-oxidation indicator to measure cell metabolic activity, making it a nondestructive assay for assessing the mitogenic activity of the bone solution described herein. Proliferation can also be assessed by measuring DNA quantification, such as by using a PicoGreen[™] DNA assay, radioactive labeling of DNA synthesis, such as [³H]thymidine labeling or BrdU incorporation. Proliferation can also be assessed via manual cell counting, such as staining cells with trypan blue and counting with a hemacytometer.

[0068] The invention also relates to methods of increasing or promoting osteogenesis, chondrogenesis, or ligament/tendon genesis in cells. The methods may comprise culturing the cells on a cell culture surface coated with the bone matrix produced by the method described herein. As used herein, “osteogenesis” is the deposition of new bone material or formation of new bone, including, but not limited to, intramembranous osteogenesis and endochondral osteogenesis. As used herein, “chondrogenesis” is the deposition new cartilage material or formation of new cartilage. As used herein, “ligament/tendon genesis” is the deposition new ligament and/or tendon material or formation of new ligament and/or tendon. The osteogenic, chondrogenic, ligament, or tendon inducing activity of the surface coated with the bone matrix may or may not be altered, including but not limited to, enhanced activity, relative to a surface without the bone matrix coating or a natural surface without any coating. The cells may include cells in any tissue in which bone, cartilage, ligament, or tendon formation is desired, such as, but not limited to, bone, cartilage, ligament, muscle, tendon, etc.

[0069] The invention also relates to methods of treating a tissue or organ defect or injury, for example, a musculoskeletal, dental or soft-tissue defect or injury, in an animal comprising administering (1) cells cultured on the surface coated with the bone matrix produced by the methods described herein and/or (2) the bone matrix implant described herein to the tissue or organ defect (e.g. osseous defects, defects in cartilage, ligament, tendon, spinal disk, and tendon insertion site to bone).

[0070] The invention further relates to methods of treating a tissue or an organ defect or injury, for example a musculoskeletal, dental or soft-tissue defect, in an animal by applying a biocompatible matrix

partially or fully coated with the bone matrix by the methods described herein to the defect, and application to the defect may be accomplished by injecting the coated biocompatible matrix into the defect, inserting the coated biocompatible matrix between tissue or organ, or placing the coated biocompatible matrix on top of the defect. The present invention is also directed to treating a defect or injury in an organ by applying a coated biocompatible matrix to the defect.

[0071] In yet another embodiment, cells may be seeded onto a biocompatible matrix coated with the bone matrix prepared by the methods provided herein. The cells seeded on the coated matrix can be any cell, such as but not limited to, osteoblasts, chondrocytes, ligament cells, tendon cells, progenitor cells, and stem cells disclosed herein or otherwise known in the art. The seeded cells may be allowed to proliferate and possibly attach to the matrix. Methods of seeding cells onto matrices, such as collagen matrix coated with the bone matrix prepared by the methods provided herein, are well known in the art.

[0072] Any of the methods of the present invention can be performed in virtually any setting, such as an in vivo, ex vivo, in situ or in vitro setting. For example, methods of promoting osteogenesis, chondrogenesis, or tendon/ligament inducing activities in cells may be performed in cell culture, may be performed in seeded cells on matrix, or may be performed in an intact organism. Moreover, any combination of any two or more of any of the embodiments described herein are contemplated.

[0073] The invention further relates to bone matrix scaffolds comprising one or more bone matrix nanofibers. In one aspect, the bone matrix scaffold may essentially consist of the bone matrix nanofibers. In another aspect, the bone matrix scaffold may consist of the bone matrix nanofibers. The bone matrix scaffold described herein may be biocompatible. A biocompatible bone matrix scaffold may be non-toxic, non-eliciting or stimulating severe inflammatory response or immunological rejections, and/or devoid of other undesired reactions at the implantation site. The bone matrix nanofiber described herein comprises bone.

[0074] The bone matrix scaffold and bone matrix nanofiber of the present invention may type I collagen. In some embodiments, total type I collagen may be present in the bone matrix scaffold and/or bone matrix nanofiber of the present invention in an amount from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, or from about 80% to about 95% by weight in it protein content. In additional embodiments, total type II collagen may be present in the bone matrix scaffold and/or bone matrix nanofiber of the present invention in an amount about 10% or less, about 5% or less, about 3% or less, or about 1% or less by weight in it protein content. In further embodiments, collagen type II may be absent from the bone matrix scaffold and/or bone matrix nanofiber of the present invention. The types of

collagen that are present or absent in the composition can be easily assessed using routine methods in the art. Methods of identifying and quantifying type of collagen are well known in the art, as disclosed, for example in Schnaper, H.W. and Kleinman, H.K., *Pediatr. Nephrol.*, 7:96-104 (1993), which is incorporated by reference.

[0075] In one aspect, the bone matrix nanofiber described herein may have less than about 8 wt%, 7 wt%, 6 wt%, 5 wt%, 4 wt%, or 3 wt% residual calcium. In another aspect, the average residual calcium amount in the bone matrix scaffold described herein may be less than about 8 wt%, 7 wt%, 6 wt%, 5 wt%, 4 wt%, or 3 wt%.

[0076] The term nanofiber as used herein means a fiber comprising a diameter of about 1000 nm or less. Relative to the parent bulk material, nano-features can impart many extraordinary properties to macrostructures, including superior mechanical, electrical, optical and magnetic properties, adding surface functionality and yielding high surface area. Matrices of nanofibers of varying diameters may show a range of variable surface properties (*e.g.* hydrophobicity and hydrophilicity), porosities, and usually superior mechanical properties (*e.g.* tensile strength, stiffness) relative to the material in other forms.

[0077] Nanofibers as matrices recently found applications for orchestrating regeneration and cellular repair processes, whereby the nano-architecture itself provides the specific developmental or morphogenetic cues. Along with proteins, growth factors, and therapeutic agents, nanofibers matrices may influence the cellular response. Further, scaffold fiber alignment plays an important biological role, such that, for example, aligned nanofibers guide cell migration, having been applied to regenerating injured neurons by guiding outgrowth. The matrix of electrospun nanofibers may mimic the natural ECM, including properties such as high porosity and high surface to volume ratios, which facilitate oxygen transport and cell waste removal, assist in cell migration across the implanted scaffold, and make delivery of therapeutic agents possible. In implanted structures for clinical use, nanofiber matrices may have further advantages of improved biocompatibility and reduced friction, and thus reduced irritation, upon delivery and as implanted.

[0078] In some embodiments, the nanofiber described herein may have an average diameter of about 1000 nm or less, 900 nm or less, 800 nm or less, 700 nm or less, 600 nm or less, 500 nm or less, 400 nm or less, 300 nm or less, 200 nm or less, 100 nm or less, 50 nm or less, 20 nm or less, or 10 nm or less. In additional embodiments, the total nanofibers in the bone matrix scaffolds described herein may have an average diameter of about 1000 nm or less, 900 nm or less, 800 nm or less, 700 nm or less, 600 nm or less,

500 nm or less, 400 nm or less, 300 nm or less, 200 nm or less, 100 nm or less, 50 nm or less, 20 nm or less, or 10 nm or less.

[0079] In one aspect, the bone matrix nanofiber described herein may have a length from about 0.1 cm to about 10 cm, from about 1 cm to about 10 cm, from about 0.1 cm to about 20 cm, from about 1 cm to about 20 cm, from about 10 cm to about 20 cm, from about 0.1 cm to about 30 cm, from about 1 cm to about 30 cm, from about 10 cm to about 30 cm, from about 20 cm to about 30 cm, from about 0.1 cm to about 40 cm, from about 1 cm to about 40 cm, from about 10 cm to about 40 cm, from about 20 cm to about 40 cm, from about 30 cm to about 40 cm, from about 0.1 cm to about 50 cm, from about 1 cm to about 50 cm, from about 10 cm to about 50 cm, from about 20 cm to about 50 cm, from about 30 cm to about 50 cm, from about 40 cm to about 50 cm, 0.1 cm to about 60 cm, from about 1 cm to about 60 cm, from about 10 cm to about 60 cm, from about 20 cm to about 60 cm, from about 30 cm to about 60 cm, or from about 40 cm to about 60 cm. In another aspect the total nanofibers in the bone matrix scaffolds described herein may have an average length from about 0.1 cm to about 10 cm, from about 1 cm to about 10 cm, from about 0.1 cm to about 20 cm, from about 1 cm to about 20 cm, from about 10 cm to about 20 cm, from about 0.1 cm to about 30 cm, from about 1 cm to about 30 cm, from about 10 cm to about 30 cm, from about 20 cm to about 30 cm, from about 0.1 cm to about 40 cm, from about 1 cm to about 40 cm, from about 10 cm to about 40 cm, from about 20 cm to about 40 cm, from about 30 cm to about 40 cm, from about 0.1 cm to about 50 cm, from about 1 cm to about 50 cm, from about 10 cm to about 50 cm, from about 20 cm to about 50 cm, from about 30 cm to about 50 cm, from about 40 cm to about 50 cm, 0.1 cm to about 60 cm, from about 1 cm to about 60 cm, from about 10 cm to about 60 cm, from about 20 cm to about 60 cm, from about 30 cm to about 60 cm, or from about 40 cm to about 60 cm.

[0080] In one aspect, the bone matrix scaffold has an average porosity from about 60% to about 70%, from about 60% to about 80%, from about 60% to about 90%, from about 60% to about 95%, from about 70% to about 80%, from about 70% to about 90%, from about 70% to about 95%, from about 80% to about 90%, or from about 80% to about 95%.

[0081] In some embodiments, the bone matrix nanofiber described herein may be prepared, for example, by electrospinning, meltblowing, bicomponent spinning, for spinning, flash-spinning, extrusion, core-sheath electrospinning with or without copolymer, or self-assembly.

[0082] Alignment of the bone matrix nanofibers in the bone matrix scaffold may be measured by a fast Fourier transform (FFT) analysis. For example, the FFT analysis may be performed by the methods described in *Measuring fiber alignment in electrospun scaffolds: a user's guide to the 2D fast Fourier*

transform approach, Ayres CE, Jha BS, Meredith H, Bowman JR, Bowlin GL, Henderson SC, Simpson DG. J Biomater Sci Polym Ed. 2008; 19(5):603-21, which is incorporated by reference in its entirety. In some embodiments, FFT result of the bone matrix nanofibers described herein may have adjacent major peaks with about 180° apart from each other.

[0083] In one aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise an accessory polymer. An “accessory polymer” refers to a polymer that may be added to the bone matrix nanofibers and have any effect on their physical, chemical, and/or biological properties (*e.g.* tensile strength, hydrophilicity, biocompatibility). For example, the accessory polymer may be selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly(lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalkanoates, starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/surfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelatin, polyphenols, levan, lignin, curd, inulin, tannin, polygalactosamine, humic acid, shellac, pullulan, poly-gamma-glutamic acid, elastin, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils.

[0084] In another aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise one or more biodegradable, biocompatible polymers. The biodegradable, biocompatible polymers may include, but is not limited to, ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. The biodegradable, biocompatible polymers may further include a number of synthetic biodegradable polymers that can serve as osteoconductive or chondroconductive biocompatible matrices with sustained release characteristics. Descriptions of these polymers can be found in Behravesh (1999) Clinical Orthopaedics 367, S118 and Lu (2000) Polymeric Delivery Vehicles for Bone Growth Factors in Controlled Drug Delivery: Designing Technologies for the Future, Park and Miron eds., American Chemical Society, which is incorporated herein in its entirety. Examples of these polymers include poly α -hydroxy esters such as polylactic acid/polyglycolic acid homopolymers and copolymers, polyphosphazenes (PPHOS), polyanhydrides and poly(propylene fumarates).

[0085] Polylactic acid/polyglycolic acid (PLGA) homo and copolymers are well known in the art as sustained release vehicles. The rate of release can be adjusted by the skilled artisan by variation of polylactic acid to polyglycolic acid ratio and the molecular weight of the polymer (see Anderson (1997) Adv. Drug Deliv. Rev. 28:5. The incorporation of PEG into the polymer as a blend to form microparticle

matrices allows further alteration of the release profile of the active ingredient (see Cleek (1997) J. Control Release 48, 259). Ceramics such as calcium phosphate and hydroxyapatite can also be incorporated into the formulation to improve mechanical qualities.

[0086] In another aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise an extracellular matrix component. For example, the extracellular matrix component may include, but is not limited to, collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, or mixtures thereof.

[0087] In another aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise a bioactive factor.

[0088] In another aspect, the bone matrix scaffold and/or the bone matrix nanofibers may further comprise one or more biocompatible material selected from the group consisting of collagen, polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalkanoates, starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen, gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, various gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin, chitosan, serum albumin, hyaluronic acid, lipids, surfactants, xanthan, acetoglycerides, waxes, dextran, emulsan, gelatin, polyphenols, levan, lignin, curd, inulin, tannin, polygalactosamine, humic acid, cellulose, shellac, pullulan, poly-gamma-glutamic acid, elastin, natural rubber, yeast glucans, synthetic polymers from natural fats and oils.

[0089] In some embodiments, the bone matrix scaffold of the present invention may further comprise a support. In additional embodiments, the bone matrix nanofibers described herein may be attached to and/or cover the support.

[0090] The invention also relates to methods of preparing the bone matrix scaffold. The method of preparing the bone matrix scaffold according to some embodiments of the present invention comprises preparing a bone matrix solution. The method of preparing a bone matrix solution according to some embodiments of the present invention comprises dissolving bone in an anhydrous solvent as described herein.

[0091] In one aspect, once the bone matrix solution is prepared, the bone matrix solution may be fabricated into nanofibers and mesh through electrospinning technologies as described in

US2010/0120115, which is incorporated by reference. Once fabricated into a scaffold, the bone matrix can be placed into cell culture environment as disclosed herein.

[0092] In another aspect, the bone matrix solution described herein may be fabricated into nanofibers and mesh using the exemplary electrospinning setup as shown in FIGURE 2. Electrospinning typically involves a polymer solution (or melt) maintained at its surface tension on the tip of a nozzle via a syringe in a pump. For example, when sufficiently high voltage is introduced (*e.g.* 15-40kV) to the polymer in solution to create a charge imbalance, the solution is drawn towards a grounded collector through the static electric field. As the polymer erupts from the needle and the assembling polymer whip through space, it may be subjected to a series of stretching and bending instabilities, resulting in plastic stretching and elongation to minimize these instabilities generated by repulsive electrostatic forces. As the polymer travels through space and rapidly thins into a fine stream, the solvent may evaporate, and the polymer may assemble into fibers, leaving dry nano- to micro-scale fibers of tailorable physical attributes on the collector.

[0093] Additionally, when high electrical potential is applied to a low viscosity polymer melt or solution, electrospraying may occur, which is typified by the polymer jet breaking down into fine droplets. It is therefore possible to produce particles or nano- to micro-sphere particles, fibers and bead-and-string type structures via this process from simply altering the solution or electrospinning apparatus setup variables. Among the variable in electrospinning that can be changed to tailor the final product, such parameters as polymer solution properties, applied electrical potential, polymer molecular weight, polymer solution flow rate, distance between spinner and collector, ambient parameters (humidity, air velocity, temperature) and motion of the collecting target may be altered to form defect-free nanofibers of controlled fiber distribution, diameter and alignment.

[0094] In some embodiments, the method of preparing the bone matrix scaffold according to some embodiments of the present invention comprises preparing a bone matrix solution by dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution at a temperature between about 0 °C and about 22 °C, and electrospinning the bone matrix solution as described herein to form the bone matrix scaffold.

[0095] In one aspect, once the bone matrix solution is prepared, the bone matrix solution may be fabricated into scaffolds through neutralizing the bone matrix solution. For example, the neutralizing the bone matrix solution may comprise titrating sodium hydroxide from the bone matrix solution into a

solution. In some embodiments, the neutralization may be performed using a neutralizing solution. The “neutralizing solution” described herein may be an acid-neutralizing solution present in an amount sufficient to raise the pH of the acidic bone matrix solution to about 7.0 to about 7.5 after mixing and/or rinsing. In another aspect, the acid-neutralizing solution may be present in an amount sufficient to raise the pH of the acidic bone matrix solution to about 6.5 to about 7.45 after mixing and/or rinsing. In another aspect, the acid-neutralizing solution may be present in an amount sufficient to raise the pH of the bone matrix solution to about 7.0 to about 7.4 after mixing and/or rinsing. In some embodiments, the acid-neutralizing solution is selected from the group consisting of a bicarbonate solution, a phosphate solution, a carbonate solution, a sodium hydroxide solution, a potassium hydroxide solution, a calcium hydroxide solution, an aluminum hydroxide solution, and any combination of these solutions.

[0096] In another aspect, the method of preparing the bone matrix scaffold described herein may further comprise transferring the bone matrix solution into an acid-resistant mold and drying the bone matrix solution. The drying may be performed by, for example, air drying, vacuum drying, heat drying, or freeze drying. In another aspect, the dried bone matrix solution may be neutralized by rinsing it with a neutralizing solution described herein. In another aspect, the method of preparing the bone matrix scaffold described herein may further comprise rinsing the dried bone matrix solution with water. In some embodiments, the bone matrix solution may comprise sodium chloride granulates.

[0097] In one aspect, the method of preparing the bone matrix scaffold according to some embodiments of the present invention may further comprise crosslinking the bone matrix solution. In some embodiments, the crosslinking may be performed by any conventional chemical crosslinking method (*e.g.* chemical reagent-promoted, chemically reactive linker-promoted and/or enzyme-promoted) and/or dehydrothermal crosslinking method (*e.g.* heat-promoted condensation), forming the covalently crosslinked bone matrix. In additional embodiments, the crosslinking comprises applying a cross-linking agent to the bone matrix solution. For example, the cross-linking agent may be selected from the group consisting of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), EDC/hyaluronic acid, genipin, and glutaraldehyde.

[0098] In another aspect, the method of preparing the bone matrix scaffold according to some embodiments of the present invention may further comprise applying an accessory polymer described herein to the bone matrix solution. In another aspect, the method of preparing the bone matrix scaffold according to some embodiments of the present invention may further comprise adding a bioactive factor described herein to the bone matrix scaffold.

[0099] The invention also relates to bone matrix implants produced by the methods of preparing the bone matrix scaffold. An “implant” refers to any object that is designed to be placed partially or wholly within a patient's body for one or more therapeutic or prophylactic purposes such as for tissue augmentation, contouring, restoring physiological function, repairing or restoring tissues damaged by disease or trauma, and/or delivering therapeutic agents to normal, damaged or diseased organs and tissues. A “bone matrix implant” refers to a medical device or implant that includes a volume replacement material for augmentation or reconstruction to replace a whole or part of a bone structure. A predetermined shape of the bone matrix implant may be varied to fit the implant site. A “bone matrix” refers to any mixture of the volume replacement materials for augmentation or reconstruction to replace a whole or part of a bone structure.

[00100] The invention also relates to methods of cell culture comprising culturing cells on a bone matrix scaffold produced by the method described herein.

[00101] For example, the invention provides for growing and/or culturing cells on a bone matrix scaffold produced by the method described herein. “Growing and/or culturing cells on a bone matrix scaffold” includes traditional cell culture methods as well as placing on a surface in any setting, such as in natural or synthetic biocompatible matrices or tissues. In some embodiments, the cells that are cultured on the bone matrix scaffold are stem cells. In further embodiments, the bone matrix scaffold can be used in *in vitro* methods for supporting cell growth and proliferation as well as for increasing osteogenesis, chondrogenesis, or ligament/tendon genesis in the stem cells cultured on the bone matrix scaffold.

[00102] Any cell described herewith may be cultured on a bone matrix scaffold produced by the method described herein for between about 15 minutes and about 4 weeks, about 2 hours and about 2 weeks, about 2 hours and about 1 week, about 2 hours and about 72 hours, about 24 hours and about 72 hours, or about 24 hours and about 96 hours, at between about 20 °C and about 40 °C or about 30°C and about 37°C, in an atmosphere containing between about 1% CO₂ and about 10% CO₂ or about 4% CO₂ and about 6% CO₂, in certain embodiments.

[00103] The invention also relates to methods of promoting osteoinductivity, with the methods comprising culturing cells on a bone matrix scaffold produced by the method described herein. The cells, prior to culture on the bone matrix scaffold of the present invention, may be undifferentiated or partially differentiated cells. The osteoinductive activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the bone matrix nanofiber described herein.

[00104] The invention also relates to methods of promoting chondroinductivity, with the methods comprising culturing cells on a bone matrix scaffold produced by the method described herein. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The chondroinductive activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the bone matrix nanofiber described herein.

[00105] The invention also relates to methods of promoting ligament/tendon differentiation, with the methods comprising culturing cells on a bone matrix scaffold produced by the method described herein. The cells, prior to culture on the bone matrix of the present invention, may be undifferentiated or partially differentiated cells. The ligament/tendon differentiation activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to other scaffolds without the bone matrix nanofiber described herein.

[00106] In one aspect, *in vitro* alkaline phosphatase assays may be used to evaluate osteoinductivity in cells cultured on the bone matrix scaffold prepared by the methods described herein. The ability of the bone matrix prepared by the methods of the present invention to stimulate or induce the alkaline phosphatase expression in an otherwise non-bone forming cells, such as myoblast (C2C12 cells), would indicate that the bone matrix of the present invention has osteoinductive activity. In these assays, cells cultured on other scaffolds without the bone matrix nanofiber described herein are used as negative controls to show that the baseline alkaline phosphatase expression on non-bone forming cells. The baseline of the osteoblastic markers in the negative control need not be zero, meaning that the cells in the negative control group may have at least some level of phenotypic marker(s). Accordingly, an “osteoinductive” bone matrix scaffold of the present invention would simply cause an increase in the osteoblastic markers in experimental cells over control grown on the other scaffolds without the bone matrix nanofiber.

[00107] Moreover, osteoinductivity, chondroinductivity, and ligament/tendon differentiation may be determined in tissue culture by investigating the ability of the bone matrix prepared by the methods of the present invention to differentiate or induce osteoblast phenotype, chondrocyte phenotype, ligament/tendon cell phenotype in cultured cells, such as primary cells, cell lines, or explants. For example, the cells may display increased production of a marker characteristic of osteoblasts and/or chondrocytes, such as alkaline phosphatase, etc. For example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the bone matrix scaffold having a bone matrix nanofiber described herein may be more than 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 times greater than the

scaffolds without the bone matrix nanofiber. In another example, the osteoinductive, chondroinductive, ligament/tendon differentiation potentials of the culture on the bone matrix scaffold and/or implant described herein may be more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 500 or even 1000 times greater than those of the scaffold and/or implant without any bone matrix nanofiber. Of course, this indicates that lower concentrations of modified growth factor, compared to unmodified growth factor are required to achieve the same effects.

[00108] Osteoinductivity, chondroinductivity, ligament/tendon differentiation, for assessing the bone, cartilage, ligament or tendon forming potential induced by the bone matrix scaffold of the present invention in a location such as muscle, may also be evaluated using a suitable animal model.

[00109] The invention also relates to methods of promoting cell attachment, proliferation or maintaining the differentiated state or preventing de-differentiation of osteoblasts, chondrocytes, ligament cells, tendon cells and/or any cell type disclosed herein with the methods comprising culturing the cells on a bone matrix scaffold produced by the method described herein. The proliferative activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to a scaffold without any bone matrix nanofiber.

[00110] Mitogenicity may be assessed by investigating cell proliferation induced by the bone matrix prepared by the methods of the present invention using various in vitro assays that measure metabolic activity, such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, alamarBlue[®] assay, and others. The alamarBlue[®] assay uses a non-cytotoxic reduction-oxidation indicator to measure cell metabolic activity, making it a nondestructive assay for assessing the mitogenic activity of the bone matrix scaffold and/or bone matrix nanofiber described herein.

[00111] The invention also relates to methods of increasing or promoting osteogenesis, chondrogenesis, or ligament/tendon genesis in cells. The methods may comprise culturing the cells on a bone matrix scaffold produced by the method described herein. The osteogenic, chondrogenic, ligament, or tendon inducing activity of the bone matrix scaffold may or may not be altered, including but not limited to, enhanced activity, relative to a scaffold without bone matrix nanofiber described herein.

[00112] The invention also relates to methods of treating a tissue or organ defect or injury, for example, a musculoskeletal, dental or soft-tissue defect or injury, in an animal comprising administering (1) cells cultured on the bone matrix scaffold produced by the methods described herein and/or (2) the bone matrix

implant described herein to the tissue or organ defect (e.g. osseous defects, defects in cartilage, ligament, tendon, spinal disk, and tendon insertion site to bone).

[00113] The invention further relates to methods of treating a tissue or an organ defect or injury, for example a musculoskeletal, dental or soft-tissue defect, in an animal by applying a bone matrix scaffold prepared by the methods described herein to the defect, and application to the defect may be accomplished by injecting the bone matrix scaffold into the defect, inserting the bone matrix scaffold between tissue or organ, or placing the bone matrix scaffold on top of the defect. The present invention is also directed to treating a defect or injury in an organ by applying a bone matrix scaffold to the defect.

[00114] In yet another embodiment, cells may be seeded onto a bone matrix scaffold prepared by the methods provided herein. The cells seeded on the bone matrix scaffold can be any cell, such as but not limited to, osteoblasts, chondrocytes, ligament cells, tendon cells, progenitor cells, and stem cells disclosed herein or otherwise known in the art. The seeded cells may be allowed to proliferate and possibly attach to the matrix. Methods of seeding cells onto matrices, such as collagen matrix coated with the bone matrix prepared by the methods provided herein, are well known in the art.

[00115] While the invention has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the invention is not restricted to the particular combinations of material and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the invention being indicated by the following claims. All references, patents and patent applications referred to in this application are herein incorporated by reference in their entirety.

[00116] The following examples are illustrative and are not intended to limit the scope of the invention described herein.

EXAMPLES

[00117] Example 1: Dissolving bone in various solvents

[00118] Demineralized bone matrix (DBM) was prepared by methods as shown in U.S. Patent No. 6,189,537 and 6,305,379, which are incorporated herein in its entirety. The demineralized bone powder was dissolved at 100mg/ml in a volatile solvent or mixture of solvents.

[00119] Demineralized bone powder was initially dissolved in TFA or from a 5/95, 10/90, 20/80, 50/50 and 100/0 ratio of organic solvents TFA/HFP, respectively, at a starting concentration of 30-150 mg/ml demineralized bone powder, with final ratios set at 20/80 TFA/HFA and 100 mg/ml for all subsequent experiments, apart from solubility assays. The bone was allowed to vigorously stir for 3-4 days at 4 °C up to 21 °C to maintain intact alpha-helical collagen, or at room temp to 37 °C to more rapidly dissolve the DBM with apparent increase in protein denaturation. This resulting brownish liquid was then further assayed for the ability to form a gel or film though evaporation of the volatile organic solvents with or without subsequent addition of water with the suspension placed at either 4 °C, room temp, or 37 °C, or through neutralizing the acidity of the liquid through strong base addition, such as sodium hydroxide, with or without a buffer system for physiological pH. DBM was found to dissolve in TFA and TFA/HPF at 4°C after an extended period of stirring, on the order of 14-28+ days.

[00120] DBM powder was dissolved in TFA/HFP, TFA/HFP/Water, and TFA/HFP. DBM was dissolved in TFA/HFP, and the solvent subsequently fully evaporated off to leave behind a semisolid colloidal suspension, or gel, or a thin film coating. The protein banding pattern present in this dissolved DBM in cooled TFA/HFP was also observed for reference with intact bands for the alpha chains of collagen prominently seen, indicating that proteins are maintained in structure and composition between the DBM powder (un-dissolved) and the DBM dissolved in the organic solvent system.

[00121] Other systems that were found to dissolve DBM entirely were a combination of TFA with isopropanol, and TFA with 2,2,2-trifluoroethanol (TFE), for which HFP is a precursor molecule. The DBM was dissolved for 2-6 days. While DBM was dissolved in a mixture of trifluoroacetic acid and isopropanol and in trifluoroacetic acid with 2,2,2-trifluoroethanol, but DBM was not dissolved in 2,2,2-trifluoroethanol and hexafluoroisopropanol even after 2 months of mixing.

[00122] A longer period on the order of 10-14 days suggested a degradation of collagen and other matrix proteins as seen on SDS gel analysis. SDS gel analysis was performed on various DBM compositions. The results suggest high homology of proteins present between dissolved DBM (20% TFA/80 %HFP; 50% TFA/50% HFP) and unprocessed DBM powder. The degradation of the DBM was observed when an equal volume of distilled water is added to the TFA/HFP mix. The results also showed the degradation of the DBM in 100% TFA solution.

[00123] Moreover, TFA/HFP/DBM mixture stirred for three days formed a gel from a fully evaporated solution of the dissolved gel re-suspended when placed in water at 37 °C, whereas TFA/HFP/DBM solution stirred for 14 days was only able to form a gel at 4 °C when the DBM was likewise evaporated

and rehydrated at placed at 4 °C, room temp, and 37 °C. TFA/HFP/DBM solution stirred over a 2-6 day period was stirred at 16-21 °C, as temperatures above room temp, as can be generated by the stirring plate generating and transferring a slight amount of heat, can potentially degrade the intact proteins. The addition of water into the solvent to dissolve the DBM resulted in radical protein degradation.

[00124] Example 2: ELISA study for growth factor detection

An ELISA assay was performed to detect BMP-2 in the dissolved DBM solution (DBM dissolved in TFA/HFP as shown in Example 2), as compared to controls and to the amount of BMP-2 released by the standard BMP-2 isolation technique of guanidine hydrochloride extraction (FIGURE 1). The DBM solution prepared as shown in Example 2 was shown to have significantly more BMP-2 released compared to the conventional extraction methods (e.g. Pietrzak et al., J Craniofac Surg., 17, 1, 2006, 84-90) with nearly 4-fold more BMP-2 released per gram of DBM.

[00125] Example 3: Preparation of Bone Matrix Scaffolds

[00126] 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) (CAS# 920-66-1) was from Acros Organics (Geel, Belgium), trifluoroacetic acid was obtained from Sigma-Aldrich (T6508, batch # 61496MK). Polycaprolactone (PCL) (B6003-1, B6003-2) was from Durect Lactone (Pelham, AL, <http://www.absorbables.com>). Bone powder was isolated from human donor bone procured by LifeNet Health, with ECM isolation procedures carried out in the laboratory of Dr. Roy Ogle. The electrospinning apparatus was designed using an Aladdin Programmable Syringe Pump from World Precision Instruments, Inc. (Sarasota, FL) and one or two adjustable high voltage power supply from Gamma High Voltage Research (Ormond Beach, FL). N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) was purchased from Thermo Scientific (Pittsburg, PA). Genipin was purchased from Wako (Richmond, VA).

[00127] Demineralized bone powder was dissolved in TFA or a TFA/HFP blend, tested as workable from a 10/90, 20/80, 50/50 and 100/0 ratio of organic solvents, respectively, at 30-150mg/ml bone powder concentrations, with final ideal paramaters set at 20/80 TFA/HFP at 100mg/ml and used for the subsequent experiments. DBM was also co-electrospun with an equal volume of polycaprolactone (PCL) that was dissolved in HPF at 100mg/ml. The bone is allowed to vigorously stir for 3-4 days at below room temp (<~21°C). This resulting brown colored solution was then loaded into the glass syringe with an 18ga stainless steel needle and secured into the syringe pump, in the device design shown in FIGURE 2. A stainless steel plate coated with aluminum foil or number 1 glass coverslips on top of the collector plate were used to collect randomly aligned nanofibers, or the fibers were collected between earthed

stainless steel posts 3-10mm apart to impart fiber alignment, with the stainless steel posts either statically mounted or rotating at around 4,000RPMs to enhance alignment and maximal thickness of the scaffold. The voltage source was used to apply a +20kV DC voltage to the positive lead, attached to the metal syringe needle, with the collector either earthed or with a 15kV DC voltage applied from a second power supply set to opposite polarity. The programmable syringe pump was set to dispense the solution at 0.50-3.50 mL/hr, ideally at 2.5ml/hr for pure DBM and 4ml/hr for DBM/PCL mixtures. After approximately 1 hour of electrospinning, the collector was replaced with a new aluminum sheet and the flow rate was changed. Concentrations of 10%, 50% , 90% and 100% demineralized bone blended with PCL were examined, as well as flow rates of 0.5mL/hr-5.75mL/hr to identify the ideal parameters (which can be affected day-to-day by relative humidity, ambient temperature, etc.). Further modifications to this arrangement have been made based on intended use, including multiple spinnerets, rotating targets of various shapes and sizes, focusing of the polymer jet by electrode modifications, and additional power source and grounding schemas.

[00128] Electrospun demineralized bone matrix and DBM/PCL blended samples were taken from each collector and mounted on aluminum stubs using carbon stickers. The samples were then coated with gold at about 50nm thickness using a sputter coater. Coated samples were imaged using a JOEL 6400 Scanning electron microscope (SEM) with Orion image processing. Representative images can be seen in FIGURE 3.

[00129] Scaffolds of pure demineralized bone nanofibers were found to dissolve when placed in aqueous solutions, including culture media, PBS and milliQ water, so 10mm scaffold punches were tested for stability after crosslinking with various chemical agents under a wide array of conditions. A solution of 0.01% genipin dissolved in ethanol at 37°C for 24-48hrs was found to be a suitable cross-linker, maintaining scaffold stability in aqueous solution. A 50 molar excess of EDC with 18hrs of cross-linking time was also found to sufficiently cross-link demineralized bone scaffold. Additionally, glutaraldehyde vapors also provided stability by lightly cross-linking the electrospun DBM nanofiber scaffold. These so called “zero length cross-linkers” altered the fibrous morphology of the electrospun scaffolds as shown in FIGURE 3, however, linkers or spacers commonly used with these cross-linkers will retain the native porous topology of the scaffolds. The average size of the electrospun fibers generated from pure demineralized bone matrix, and as blended with PCL, and as compared to the native demineralized bone power size was calculated by measuring 50 unique points in ImageJ64 (NIH shareware), with results shown in FIGURE 4.

[00130] To confirm co-polymerization of demineralized bone and PCL in the forming blended nanofibers of the two materials, FTIR analysis was used to assess the chemical bond structure and to quantitatively determine the presence of each polymer in the different blends. FTIR also was used to show a high degree of similarity between the unprocessed demineralized bone powder and the electrospun nanofibrous mesh as shown in FIGURE 5, where peaks/bonds were highly conserved between raw and electrospun DBM, including putative peaks that suggest a maintained alpha helical structure in the collagen present. Lastly, to assess the structure of collagen to be unchanged from the native DBM powder state we compared native DBM and electrospun DBM with pure collagen type I that was either treated or not treated overnight with pepsin to show the maintenance of the alpha 1 and alpha 2 bands of collagen type 1 between samples (FIGURE 6). The porosity of the electrospun scaffolds was characterized as shown in FIGURE 7.

[00131] Example 4: Cell Culture on Bone Matrix Scaffolds

[00132] Excellent and rapid mesenchymal stem cell attachment was also demonstrated on electrospun DBM as cross-linked with EDC or blended with PCL as compared to controls are also shown FIGURE 8.

[00133] Example 5: *In Vivo* Study

[00134] Animal studies with implanted EDC cross-linked DBM and DBM/PCL indicated new bone formation along with high cellularity in electrospun DBM-EDC cross-linked samples with a mild inflammatory response at 4 weeks of gestation of 0.1cc of material in a rat pouch assay (FIGURE 9).

[00135] Example 6: Cell Differentiation on Bone Matrix Scaffolds

[00136] Alizarin Red S histological stains of electrospun DBM/PCL, PCL and DBM indicated that trace calcium in the native DBM remains present in the electrospun variants of DBM and DBM/PCL (FIGURE 10), with the trace calcium believed critical for new bone formation. Toluidine blue histology, along with apparent bone nodule formation, confirmed osteoblastogenesis on samples of electrospun DBM that was dissolved in contact with stem cells grown on plastic (FIGURE 10).

[0001] The osteoinductive capacity of the electrospun DBM was further confirmed through showing adipose derived stem cells (ASCs) and dental pulp stem cells (DPSCs) were induced to osteoblasts from their contact with the electrospun DBM. Osteoblastogenesis was confirmed using fluorescent microscopy to visualize expression of bone sialo protein I (aka osteopontin) and bone sialo protein II using the respective monoclonal antibodies conjugated to alexa-488 secondary antibodies, with a DAPI stain for the

nucleus (FIGURE 11) all seen in cells on DBM scaffolds only. The alexa488 fluorescence was absent in control samples and on no-secondary-antibody controls.

CLAIMS

1. A method of preparing a bone matrix solution, the method comprising
dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution at a temperature lower than about 25 °C.
2. The method according to claim 1, wherein the bone is demineralized bone (DBM).
3. The method according to any of claims 1-2, wherein the bone comprises an osteogenic growth factor.
4. The method according to any of claims 1-3, wherein the bone comprises an extracellular matrix selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, and mixtures thereof.
5. The method according to any of claims 1-4, wherein the bone comprises collagen type I.
6. The method according to any of claims 1-5, wherein the bone matrix solution comprises collagen in its native triple helical structure.
7. The method according to any of claims 1-6, wherein the solvent consists of (i) the HFP, the TFE, or the isopropanol, and (ii) the TFA.
8. The method according to any of claims 1-7, wherein the solvent comprises at least about 20 wt% TFA.
9. The method according to any of claims 1-8, wherein the solvent comprises at least about 50 wt% TFA.
10. The method according to any of claims 1-9, wherein the bone is dissolved at a temperature lower than about 24 °C.
11. The method according to any of claims 1-10, wherein the bone is dissolved at a temperature lower than about 22 °C.
12. The method according to any of claims 1-11, wherein the bone is dissolved at a temperature lower than about 15 °C.
13. The method according to any of claims 1-12, wherein the bone is dissolved at a temperature lower than about 10 °C.
14. The method according to any of claims 1-13, wherein the bone is dissolved in a concentration between about 30 mg/mL and about 150 mg/mL.
15. The method according to any of claims 1-14, wherein the bone is dissolved by stirring for more than about 24 hours.
16. The method according to any of claims 1-15, wherein the bone is in the form of particles and/or powder.
17. A method of coating bone matrix on a surface, the method comprising:

dissolving a bone at a temperature lower than about 25 °C in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE), and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution,

exposing the bone matrix solution to conditions sufficient to evaporate a portion of the solvent to form a colloidal gel,

coating the surface with the colloidal gel, and

exposing the coated surface to conditions sufficient to further evaporate an amount of solvent.

18. The method according to claim 17, wherein the surface is of a second biocompatible matrix.

19. The method according to any of claims 17-18, wherein the bone comprises an osteogenic growth factor.

20. The method according to any of claims 17-19, wherein the bone comprises an extracellular matrix selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialo protein, osteopontin, and mixtures thereof.

21. The method according to any of claims 17-20, wherein the bone comprises collagen type I.

22. The method according to any of claims 17-21, wherein the dissolved bone comprises collagen in its native triple helical structure.

23. The method according to any of claims 17-22, wherein the solvent consists of (i) HFP or TFE, and (ii) TFA.

24. The method according to any of claims 17-23, wherein the solvent comprises at least about 20 wt% TFA.

25. The method according to any of claims 17-24, wherein the solvent comprises at least about 50 wt% TFA.

26. The method according to any of claims 17-25, wherein the bone is dissolved at a temperature lower than about 24 °C.

27. The method according to any of claims 17-26, wherein the bone is dissolved at a temperature lower than about 22 °C.

28. The method according to any of claims 17-27, wherein the bone is dissolved at a temperature lower than about 15 °C.

29. The method according to any of claims 17-28, wherein the bone is dissolved at a temperature lower than about 10 °C.

30. The method according to any of claims 17-29, wherein the bone is dissolved in a concentration between about 30 mg/mL and about 150 mg/mL.

31. The method according to any of claims 17-30, wherein the bone is dissolved by stirring for more than about 24 hours.

32. The method according to any of claims 17-31, wherein the bone is in the form of particles and/or powder.
33. The method according to any of claims 17-32, wherein the colloidal gel is placed in a mold to form the predetermined shape.
34. The method according to any of claims 17-33, wherein the evaporation is performed by air drying, vacuum drying, heat drying, or freeze drying.
35. The method according to any of claims 17-34, wherein the amount of the solvent to form a colloidal gel from the bone matrix solution according to some embodiments of the present invention may be between about 95 wt% and about 5 wt%.
36. The method according to any of claims 17-35, further comprising adding a bioactive factor to the bone matrix solution.
37. The method of claim 36, wherein the bioactive factor is selected from the group consisting of an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor(TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein, erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), or an interleukin, among others. The bioactive factor may be a BMP, PDGF, FGF, VEGF, TGF, insulin, among others. Examples of BMPs include but are not limited to BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and a mixture thereof.
38. A bone matrix implant produced by the method according to any of claims 17-37.
39. A synthetic surface coated with the bone matrix produced by the method according to any of claims 17-37.
40. A method of cell culture comprising culturing cells on a cell culture surface coated with the bone matrix produced by the method according to any of claims 17-37.
41. The method of claim 40, wherein the cells are selected from the group consisting of stem cells, adipose derived stem cells and dental pulp stem cells.
42. A method of promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on a surface coated with the bone matrix produced by the method according to any of claims 17-37.
43. The method of claim 42, wherein the cells are progenitor cells or adult stem cells.

44. The method of claim 43, wherein the progenitor cells or the adult stem cells are derived from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.
45. The method of claim 42, wherein the stem cells are pluripotent stem cells.
46. The method of claim 42, wherein the stem cells are selected from the group consisting of adipose derived stem cells and dental pulp stem cells.
47. A bone matrix scaffold comprising one or more bone matrix nanofibers.
48. The bone matrix scaffold of claim 47, wherein the bone matrix scaffold essentially consists of the bone matrix nanofibers.
49. The bone matrix scaffold according to any of claims 47-48, wherein the bone matrix scaffold consists of the bone matrix nanofibers.
50. The bone matrix scaffold according to any of claims 47-49, wherein the bone matrix scaffold is biocompatible.
51. The bone matrix scaffold according to any of claims 47-50, wherein the bone matrix nanofibers comprise type I collagen in its native triple helical structure.
52. The bone matrix scaffold according to any of claims 47-51, wherein the bone matrix nanofibers has less than about 8 wt% residual calcium.
53. The bone matrix scaffold according to any of claims 47-52, wherein the bone matrix nanofibers have an average diameter from about 5 nm to about 1000 nm.
54. The bone matrix scaffold according to any of claims 47-53, wherein the bone matrix nanofiber have an average diameter of less than about 100 nm.
55. The bone matrix scaffold according to any of claims 47-54, wherein the bone matrix nanofibers have an average length from about 1 cm to about 50 cm.
56. The bone matrix scaffold according to any of claims 47-55, wherein the bone matrix scaffold has an average porosity from about 60% to about 90%.
57. The bone matrix scaffold according to any of claims 47-56, wherein the bone matrix nanofibers are electrospun.
58. The bone matrix scaffold according to any of claims 47-57, wherein a fast Fourier transform (FFT) analysis result of the bone matrix nanofibers have adjacent major peaks with about 180° apart from each other.
59. The bone matrix scaffold according to any of claims 47-58, wherein the bone matrix nanofibers further comprise an accessory polymer.

60. The bone matrix scaffold of claim 59, wherein the accessory polymer is selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalkanoates, starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/surfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelatin, polyphenols, levan, lignin, curd, inulin, tannin, polygalactosamine, humic acid, shellac, pullulan, poly-gamma-glutamic acid, elastin, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils.

61. The bone matrix scaffold according to any of claims 47-60, wherein the bone matrix nanofibers further comprise one or more biodegradable, biocompatible polymers selected from the group consisting of ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid.

62. The bone matrix scaffold according to any of claims 47-61, wherein the bone matrix nanofibers further comprise an extracellular matrix component selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialoprotein, osteopontin, and mixtures thereof.

63. The bone matrix scaffold according to any of claims 47-62, wherein the bone matrix nanofibers further comprise a bioactive factor.

64. The bone matrix scaffold of claim 63, wherein the bioactive factor is selected from the group consisting of an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialoprotein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor (TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein, erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), and an interleukin.

65. The bone matrix scaffold of claim 63, wherein the bioactive factor is an osteogenic growth factor.

66. The bone matrix scaffold according to any of claims 47-65, wherein the bone matrix nanofibers further comprise one or more biocompatible material selected from the group consisting of collagen, polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalkanoates, starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen, gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, various gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin, chitosan, serum

albumin, hyaluronic acid, lipids, surfactants, xanthan, acetoglycerides, waxes, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, cellulose, shellac, pullulan, poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, synthetic polymers from natural fats and oils.

67. The bone matrix scaffold according to any of claims 47-66, further comprising a support, wherein the bone matrix nanofibers cover the support.

68. A method of preparing a bone matrix scaffold, the method comprising

dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form a bone matrix solution, and

electrospinning the bone matrix solution to form the bone matrix scaffold.

69. The method of claim 68, further comprising crosslinking the bone matrix solution.

70. The method of claim 69, wherein the crosslinking is performed by dehydrothermal crosslinking.

71. The method of claim 69, wherein the crosslinking comprises applying a cross-linking agent to the bone matrix solution.

72. The method of claim 71, wherein the cross-linking agent is selected from the group consisting of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), EDC/hyaluronic acid, genipin, and glutaraldehyde.

73. The method according to any of claims 68-72, further comprising applying an accessory polymer to the bone matrix solution.

74. The method of claim 73, wherein the accessory polymer is selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalkanoates, starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/surfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelian, polyphenols, levan, lignin, curd, ian, tannin, polygalactosamine, humic acid, shellac, pullulan, poly-gamma-glutamic acid, elsinan, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils.

75. The method according to any of claims 68-74, wherein the bone is demineralized bone (DBM).

76. The method according to any of claims 68-75, wherein the bone comprises an osteogenic growth factor.

77. The method according to any of claims 68-76, wherein the bone comprises an extracellular matrix selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialoprotein, osteopontin, and mixtures thereof.

78. The method according to any of claims 68-77, wherein the bone comprises collagen type I.
79. The method according to any of claims 68-78, wherein the bone matrix solution comprises collagen in its native triple helical structure.
80. The method according to any of claims 68-79, wherein the anhydrous solvent consists of (i) the HFP, the TFE, or the isopropanol, and (ii) the TFA.
81. The method according to any of claims 68-80, wherein the anhydrous solvent comprises at least about 20 wt% TFA.
82. The method according to any of claims 68-81, wherein the anhydrous solvent comprises at least about 50 wt% TFA.
83. The method according to any of claims 68-82, wherein the bone is dissolved at a temperature lower than about 25 °C.
84. The method according to any of claims 68-83, wherein the bone is dissolved at a temperature lower than about 24 °C.
85. The method according to any of claims 68-84, wherein the bone is dissolved at a temperature lower than about 22 °C.
86. The method according to any of claims 68-85, wherein the bone is dissolved at a temperature lower than about 15 °C.
87. The method according to any of claims 68-86, wherein the bone is dissolved at a temperature lower than about 10 °C.
88. The method according to any of claims 68-87, wherein the bone is dissolved in a concentration between about 30 mg/mL and about 150 mg/mL.
89. The method according to any of claims 68-88, wherein the bone is dissolved by stirring for more than about 24 hours.
90. The method according to any of claims 68-89, wherein the bone is in the form of particles and/or powder.
91. The method according to any of claims 68-90, further comprising adding a bioactive factor to the bone matrix scaffold.
92. The method of claim 91, wherein the bioactive factor is selected from the group consisting of an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor(TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein,

erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), or an interleukin, among others. The bioactive factor may be a BMP, PDGF, FGF, VEGF, TGF, insulin, among others. Examples of BMPs include but are not limited to BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and a mixture thereof.

93. A bone matrix implant produced by the method according to any of claims 68-92.

94. A method of preparing a bone matrix scaffold, the method comprising

dissolving bone in an amount of an anhydrous solvent comprising (i) hexafluoroisopropanol (HFP) or 2, 2, 2-trifluoroethanol (TFE) and (ii) at least about 5 wt% trifluoroacetic acid (TFA) sufficient to form the bone matrix solution, and

neutralizing the bone matrix solution to form the bone matrix scaffold.

95. The method of claim 94, further comprising crosslinking the bone matrix solution.

96. The method of claim 95, wherein the crosslinking is performed by dehydrothermal crosslinking.

97. The method of claim 96, wherein the crosslinking comprises applying a cross-linking agent to the bone matrix solution.

98. The method of claim 97, wherein the cross-linking agent is selected from the group consisting of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), EDC/hyaluronic acid, genipin, and glutaraldehyde.

99. The method according to any of claims 94-98, further comprising applying an accessory polymer to the bone matrix solution.

100. The method of claim 99, wherein the accessory polymer is selected from the group consisting of polycaprolactone, poly(glycolic acid), poly(lactic acid), polydioxanone, poly (lactide-co-glycolide) copolymers, polyesters polysaccharides, polyhydroxyalkanoates, starch, polylactic acid, cellulose, proteins, agar, silks, alginate, collagen/gelatin, carrageenan, elastin, pectin, resilin, konjac, adhesives, gums, polyamino acids, polysaccharides, soy, zein, wheat gluten, casein, chitin/chitosan, serum albumin, hyaluronic acid, lipids/surfactants, xanthan, acetoglycerides, waxes, surfactants, dextran, emulsan, gelatin, polyphenols, levan, lignin, curd, inulin, tannin, polygalactosamine, humic acid, shellac, pullulan, poly-gamma-glutamic acid, elastin, natural rubber, yeast glucans, and synthetic polymers from natural fats and oils.

101. The method according to any of claims 94-100, wherein the bone is demineralized bone (DBM).

102. The method according to any of claims 94-101, wherein the bone comprises an osteogenic growth factor.

103. The method according to any of claims 94-102, wherein the bone comprises an extracellular matrix selected from the group consisting of collagen, glycosaminoglycans, osteocalcin, osteonectin, bone sialoprotein, osteopontin, and mixtures thereof.

104. The method according to any of claims 94-103, wherein the bone comprises collagen type I.
105. The method according to any of claims 94-104, wherein the bone matrix solution comprises collagen in its native triple helical structure.
106. The method according to any of claims 94-105, wherein the anhydrous solvent consists of (i) the HFP, the TFE, or the isopropanol, and (ii) the TFA.
107. The method according to any of claims 94-106, wherein the anhydrous solvent comprises at least about 20 wt% TFA.
108. The method according to any of claims 94-107, wherein the anhydrous solvent comprises at least about 50 wt% TFA.
109. The method according to any of claims 94-108, wherein the bone is dissolved at a temperature lower than about 25 °C.
110. The method according to any of claims 94-109, wherein the bone is dissolved at a temperature lower than about 24 °C.
111. The method according to any of claims 94-110, wherein the bone is dissolved at a temperature lower than about 22 °C.
112. The method according to any of claims 94-111, wherein the bone is dissolved at a temperature lower than about 15 °C.
113. The method according to any of claims 94-112, wherein the bone is dissolved at a temperature lower than about 10 °C.
114. The method according to any of claims 94-113, wherein the bone is dissolved in a concentration between about 30 mg/mL and about 150 mg/mL.
115. The method according to any of claims 94-114, wherein the bone is dissolved by stirring for more than about 24 hours.
116. The method according to any of claims 94-115, wherein the bone is in the form of particles and/or powder.
117. The method according to any of claims 94-116, further comprising adding a bioactive factor to the bone matrix scaffold.
118. The method of claim 117, wherein the bioactive factor is selected from the group consisting of an osteogenic growth factor, collagen, glycosaminoglycans, osteonectin, bone sialo protein, an osteoinductive factor, a chondrogenic factor, a cytokine, a mitogenic factor, a chemotactic factor, a transforming growth factor(TGF), a fibroblast growth factor (FGF), an angiogenic factor, an insulin-like growth factor (IGF), a platelet-derived growth factor (PDGF), an epidermal growth factor (EGF), a vascular endothelial growth factor (VEGF), a nerve growth factor (NGF), a neurotrophin, a bone morphogenetic protein (BMP), osteogenin, osteopontin, osteocalcin, cementum attachment protein,

erythropoietin, thrombopoietin, tumor necrosis factor (TNF), an interferon, a colony stimulating factor (CSF), or an interleukin, among others. The bioactive factor may be a BMP, PDGF, FGF, VEGF, TGF, insulin, among others. Examples of BMPs include but are not limited to BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, and a mixture thereof.

119. The method according to any of claims 94-118, wherein the neutralizing the bone matrix solution comprises titrating sodium hydroxide from the bone matrix solution into a solution.

120. The method according to any of claims 94-119, further comprising
transferring the bone matrix solution into an acid-resistant mold,
drying the bone matrix solution, and
neutralizing the dried bone matrix solution by rinsing with a neutralizing solution.

121. The method of claim 120, further comprising rinsing the dried bone matrix solution with water.

122. The method of claim 121, wherein the bone matrix solution comprises sodium chloride granulates.

123. The method according to any of claims 120-122, wherein the drying is performed by air drying.

124. A bone matrix implant produced by the method according to any of claims 94-123.

125. A method of cell culture comprising culturing cells on the bone matrix scaffold according to any of claims 47-67 in condition suitable for the cell culture.

126. The method of claim 125, wherein the cells are selected from the group consisting of stem cells, adipose derived stem cells and dental pulp stem cells.

127. A method of promoting differentiation of stem cells into osteoblasts, chondrocytes, ligament or tendon, the method comprising culturing the cells on the bone matrix scaffold according to any of claims 47-67 in conditions suitable for the cell differentiation.

128. The method of claim 127, wherein the stem cells are progenitor cells or adult stem cells.

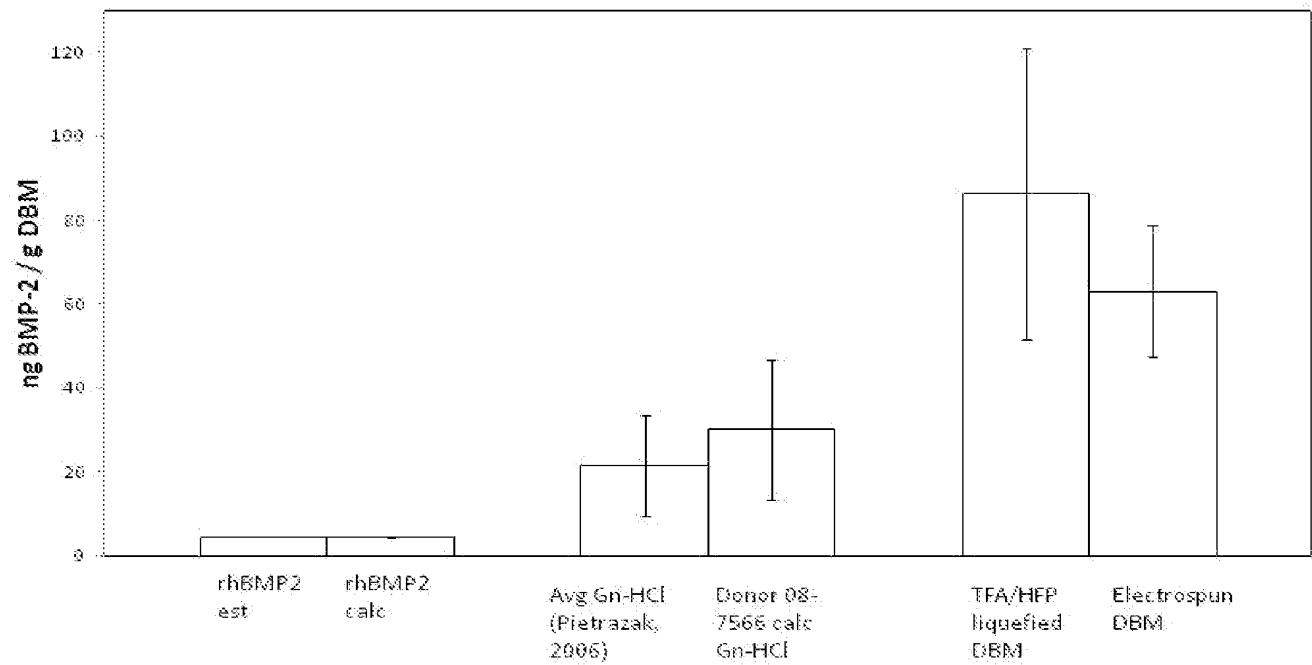
129. The method of claim 128, wherein the progenitor cells or the adult stem cells are derived or isolated from placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, periosteum, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, or muscle.

130. The method of claim 127, wherein the stem cells are pluripotent stem cells.

131. The method of claim 127, wherein the stem cells are selected from the group consisting of adipose derived stem cells and dental pulp stem cells.

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FIGURE 1.



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FIGURE 2.

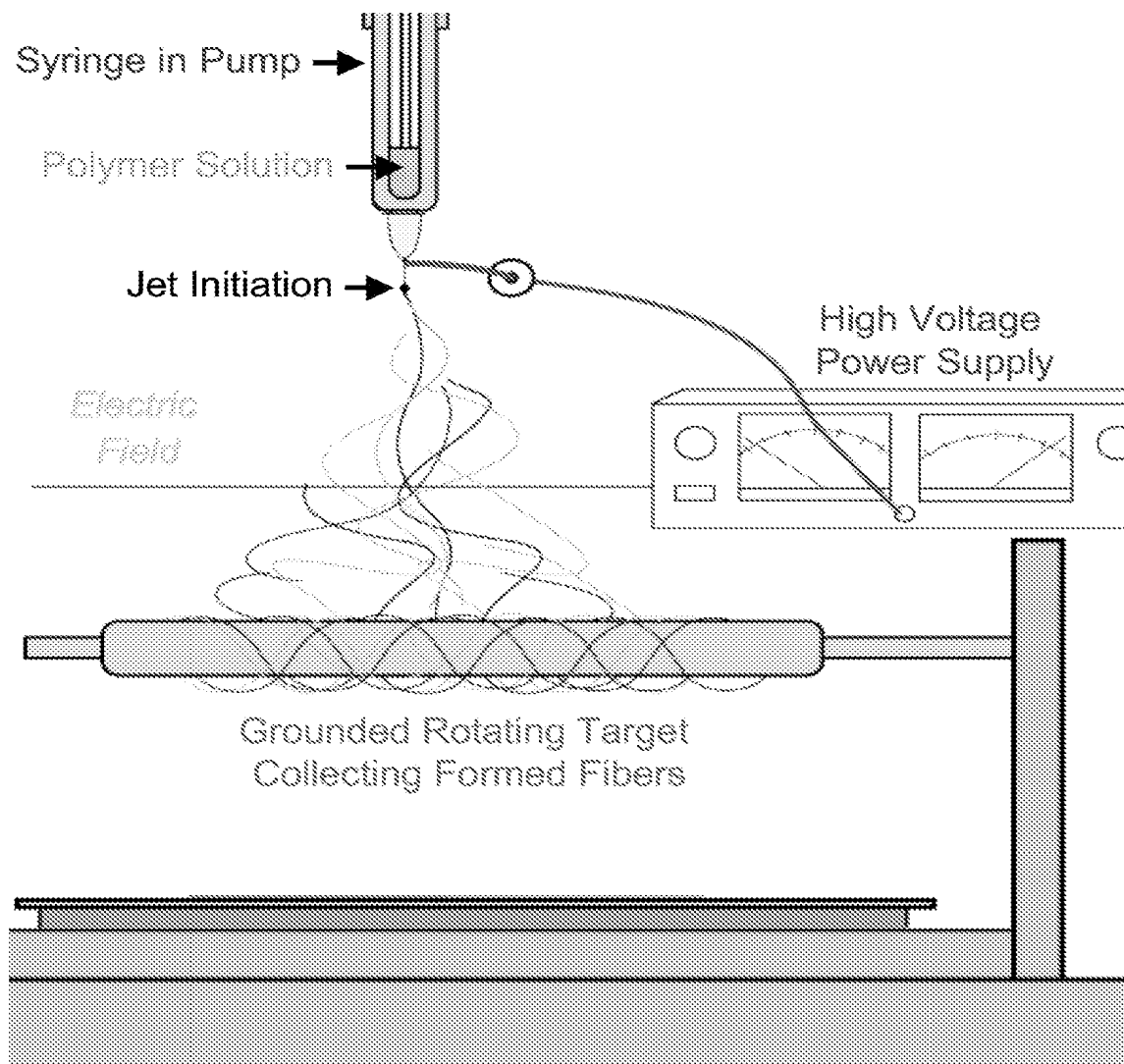
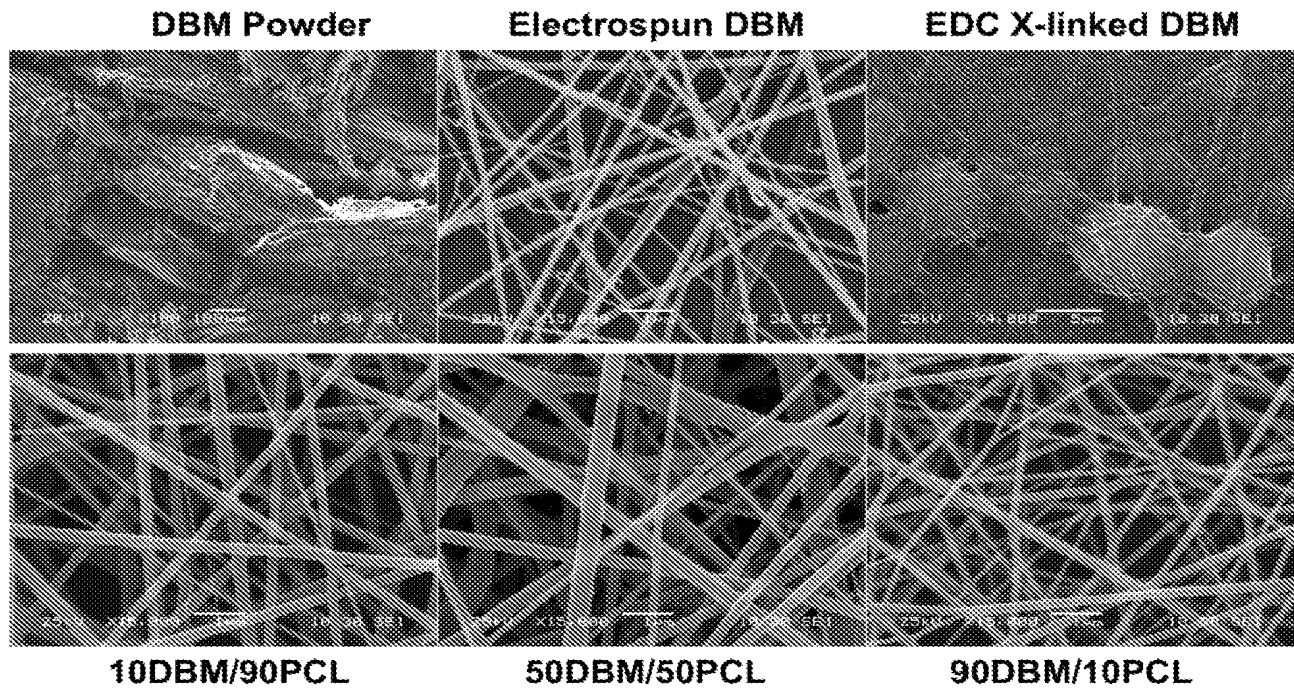
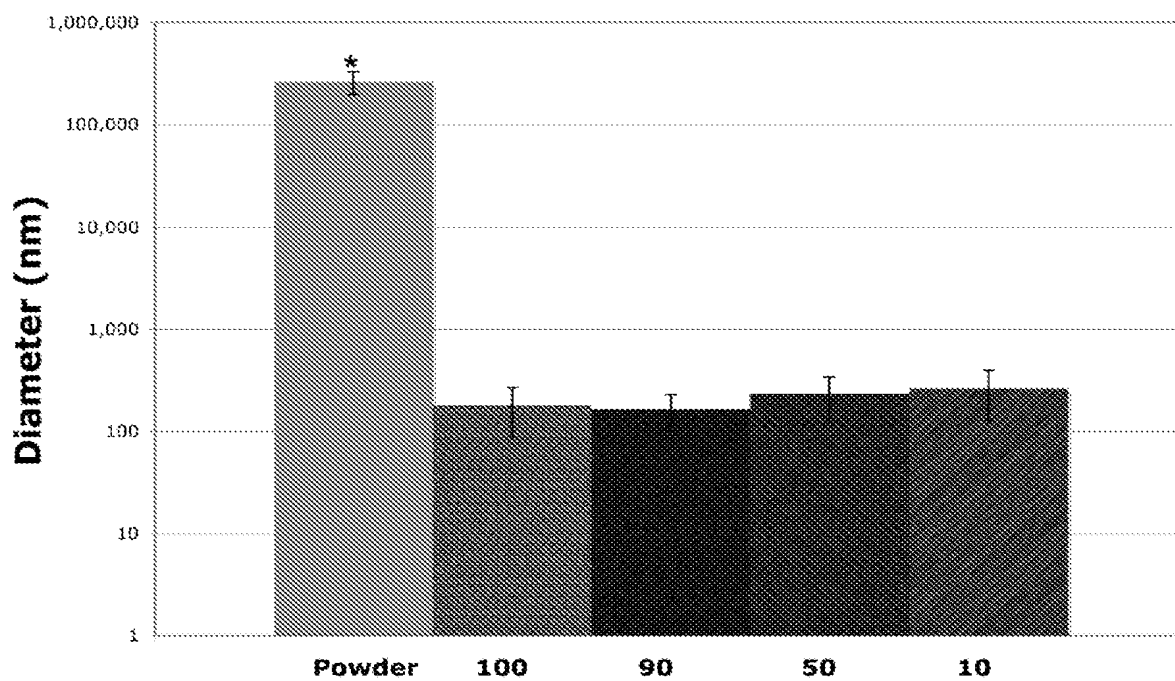


FIGURE 3.



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FIGURE 4.



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FIGURE 5.

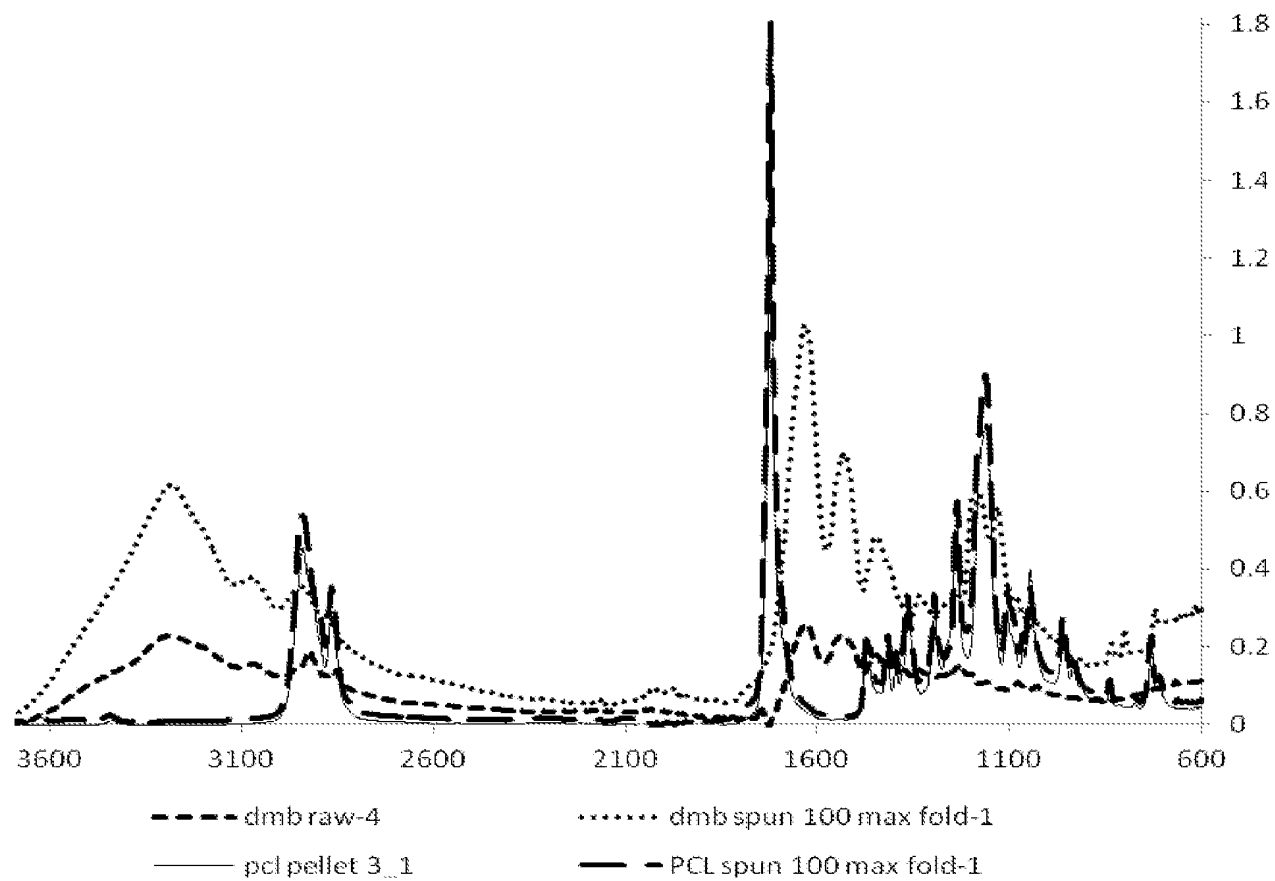
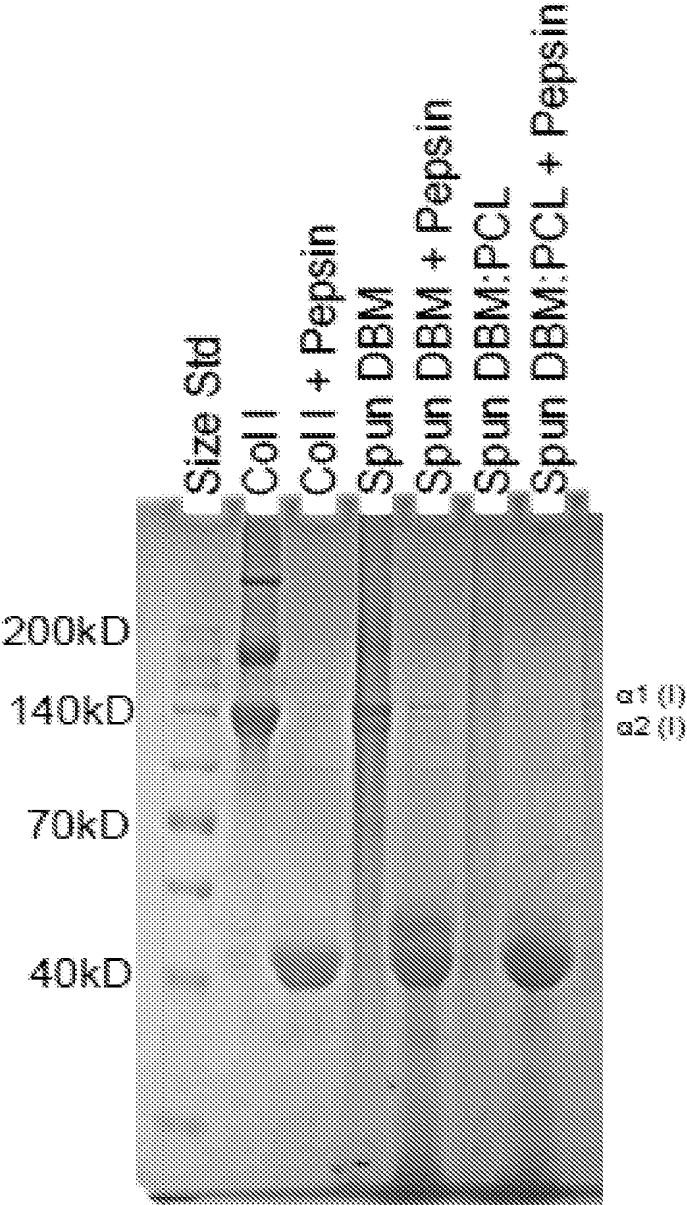
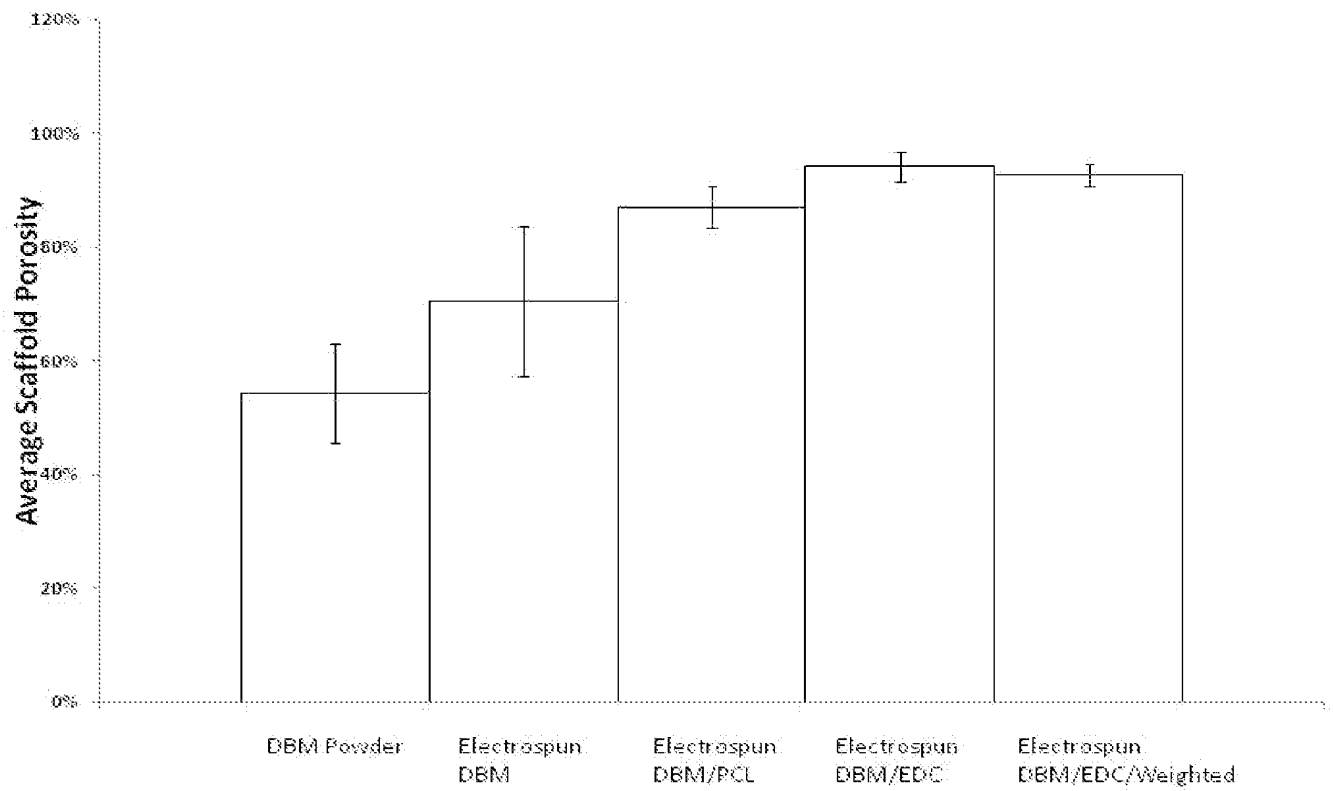


FIGURE 6.



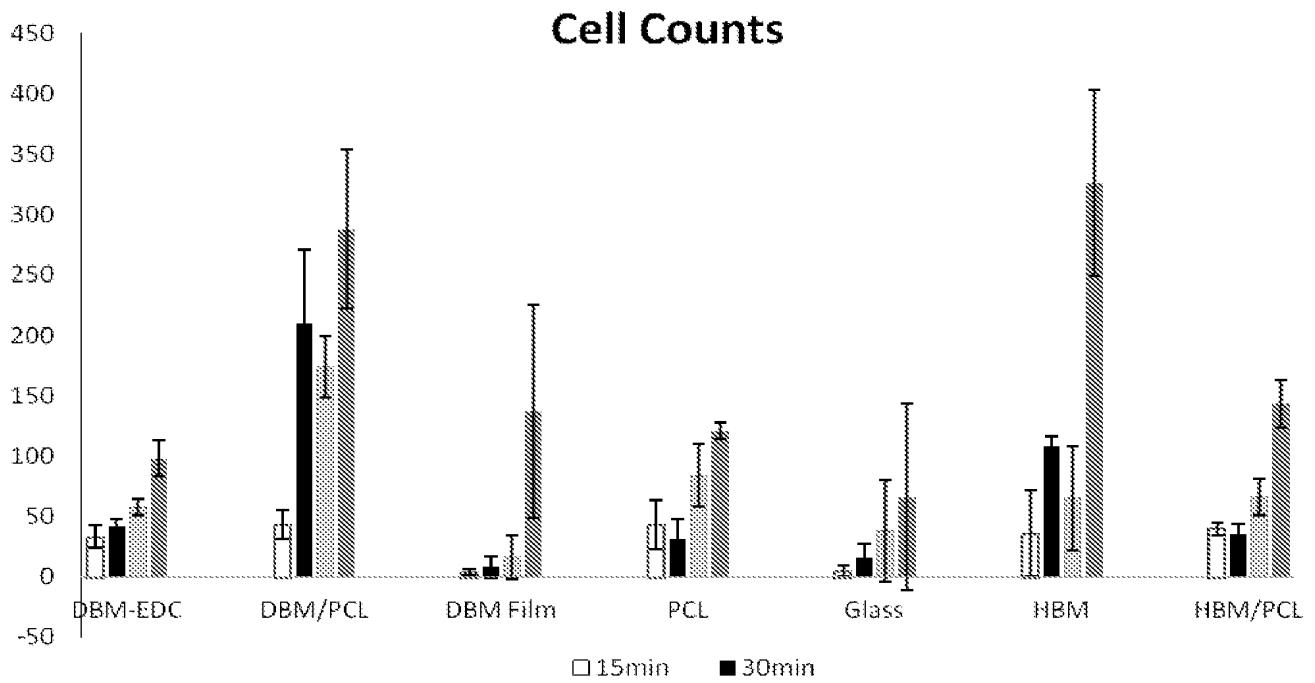
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FIGURE 7.



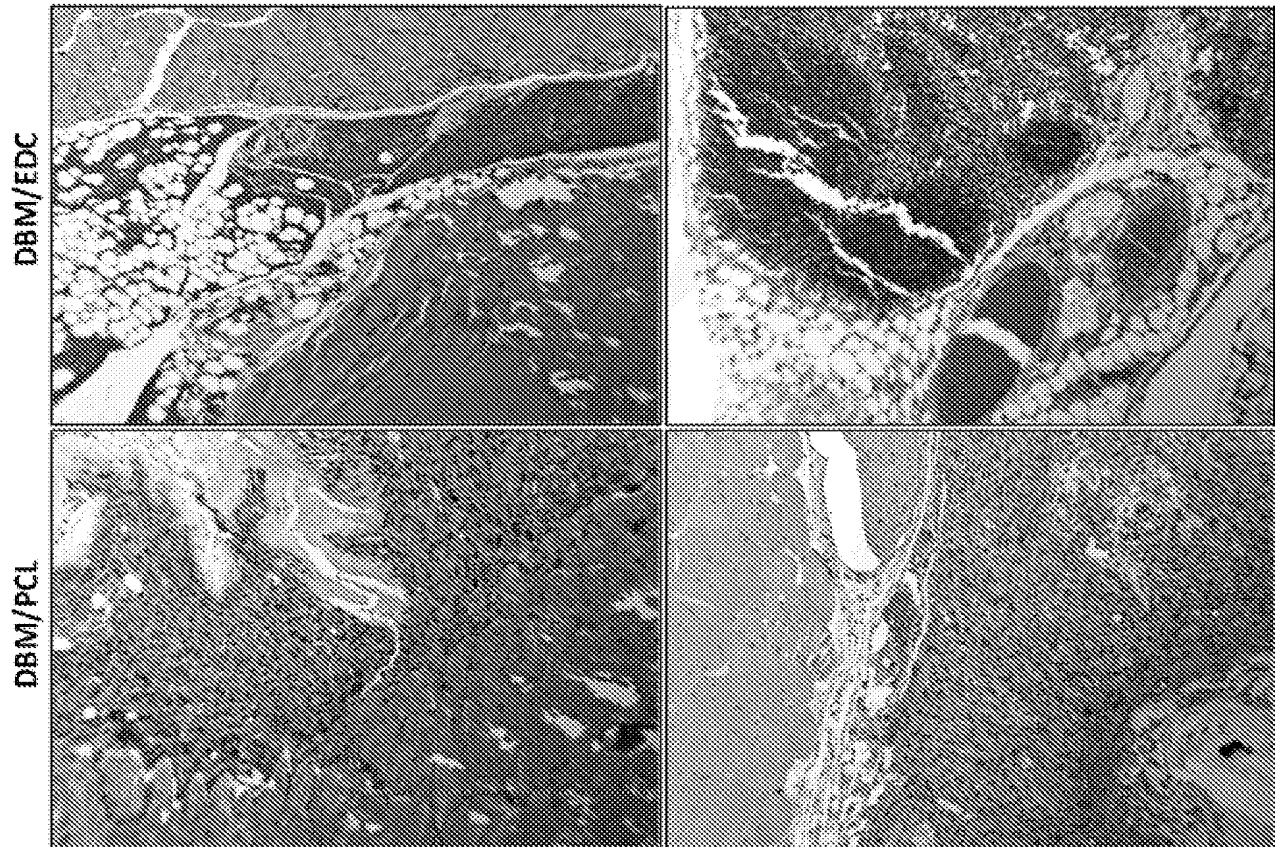
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FIGURE 8.



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FIGURE 9.



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FIGURE 10.

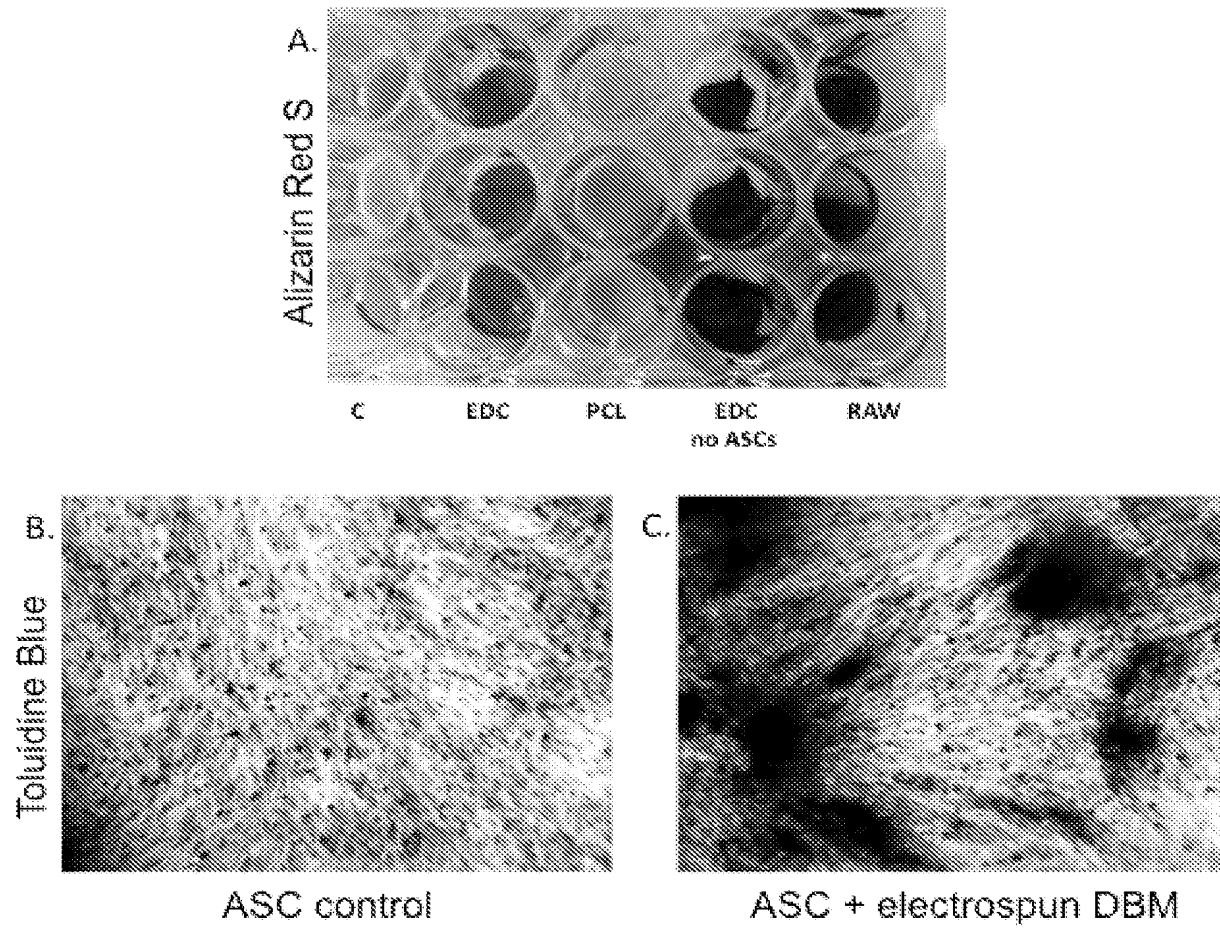
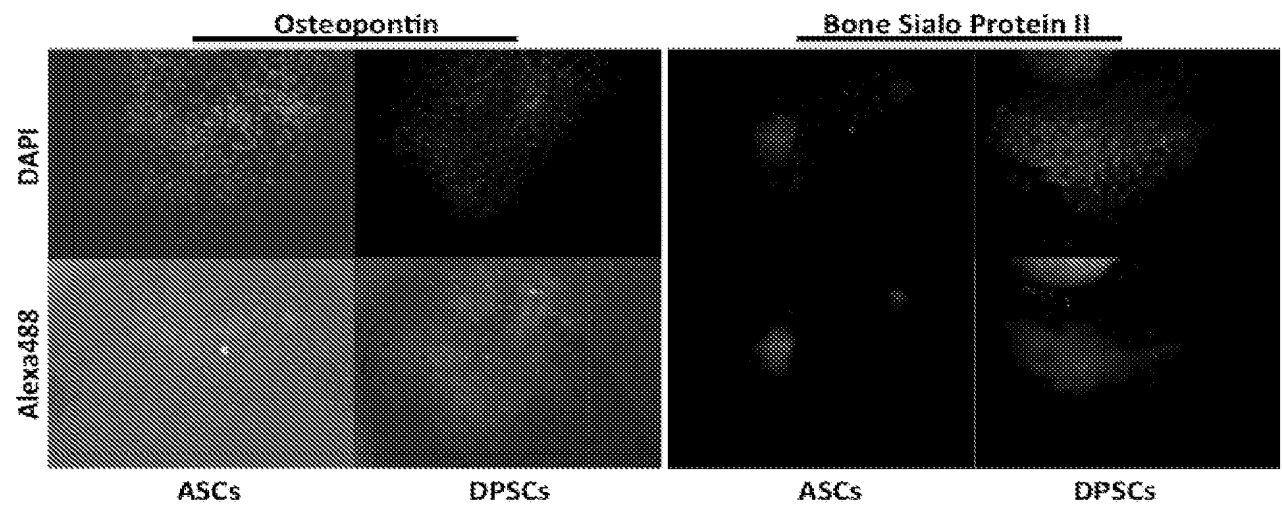


FIGURE 11.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/25226

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/32 (2013.01)

USPC - 424/549

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 424/549

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 424/549, 426, 602 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST, Google Patents, Google Scholar, Freepatentsonline, PatBase: Bone Matrix, Dissolving bone, demineralized bone, DBM, Liquefaction, Anhydrous fluoroalcohols, HFIP, HFP, TFE, Hexafluoroisopropanol, Trifluoroethanol, TFA, Dehydrothermal crosslinking, Nanofibers, implant\$, Demineral\$, colloid\$, colloid#, dissol#.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- A	WO 2009/082554 A2 (Wei et al.) 02 July 2009 (02.07.2009) para [0008]-[0009], [0033], [0049], [0077]-[0079], [0105]-[0107], [0119]-[0121].	47-49 ----- 17-19
A	US 6340477 B1 (Anderson) 22 January 2002 (22.01.2002) col. 1, ln 34-65; col. 3, ln 5-25.	1-3
A	US 2011/0218646 A1 (Pertici) 08 September 2011 (08.09.2011) para [0012], [0031], [0039]-[0040], [0060]-[0061].	68-75, 94-100

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 20 March 2013 (20.03.2013)	Date of mailing of the international search report 23 APR 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 13/25226

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-16, 20-46, 50-67, 76-93, 101-131
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 February 2010 (11.02.2010)



(10) International Publication Number
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(26) Publication Language: English

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(72) Inventors; and

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(74) Agent: **TENG, Sally, P.**; Morgan, Lewis & Bockius LLP, 1111 Pennsylvania Avenue, Nw, Washington, DC 20004 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: COMPOSITION FOR A TISSUE REPAIR IMPLANT AND METHODS OF MAKING THE SAME

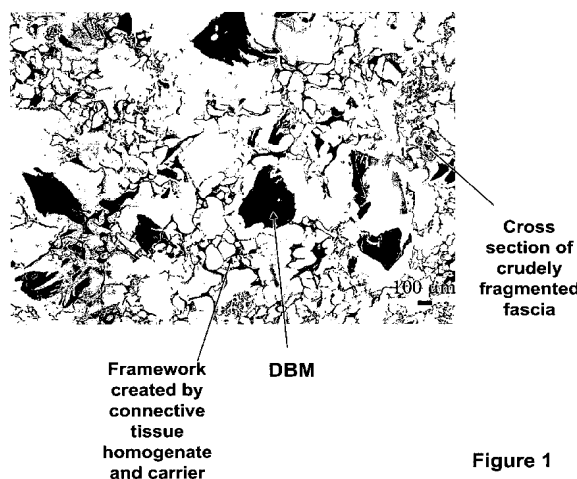


Figure 1

(57) Abstract: The invention is directed to a process for making a tissue repair implant having a porous sponge-like structure to repair bone, cartilage, or soft tissue defects by producing a connective tissue homogenate from one or more connective tissues, mixing the connective tissue homogenate with a carrier solution to produce a connective tissue carrier, optionally mixing one or more natural or synthetic bone fragments with said connective tissue carrier to produce a tissue repair mixture, freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework to entrap the natural or synthetic bone fragments, treating the frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure. A crudely fragmented connective tissue from one or more connective tissues is optionally mixed with the tissue repair mixture before freezing or freeze-drying.

WO 2010/016942 A1

Composition for a Tissue Repair Implant and Methods of Making the Same

Inventors: Silvia S. Chen, Jingsong Chen, and Lloyd Wolfinbarger

RELATED APPLICATION

[0001] The present invention claims priority to U.S. Application 12/188,127, filed August 7, 2009, which is incorporated herein in its entirety.

FIELD OF THE INVENTION

[0002] The present invention is related to a tissue repair implant having a porous sponge-like structure and to methods of preparing such an implant. The present invention is also related to methods of using the implant to repair defective tissues.

BACKGROUND OF THE INVENTION

[0003] The ability to promote tissue regrowth in vivo can facilitate wound healing and post-surgical recovery of patients who have suffered tissue damage or destruction. The need for such methods and compositions is readily apparent, when considering that in 1999, approximately 500,000 bone graft procedures were performed in the United States alone. Ideal bone graft materials for use in such procedures possess characteristics necessary to new bone growth, namely osteoconductivity and osteoinductivity.

[0004] Osteoconductivity refers to a graft's ability to support the attachment of new osteoblasts and osteoprogenitor cells. The osteoconductive components of a graft provide an interconnected structure through which new cells can migrate and new blood vessels can form. Osteoinductivity refers to the ability of a graft to induce nondifferentiated stem cells or osteoprogenitor cells to differentiate into osteoblasts.

[0005] In 1998, 9 of 10 bone graft procedures performed in the United States involved the use of either autograft or allograft bone tissue. Despite the benefits of autografts and allografts, the limitations of each have necessitated the pursuit of alternative graft materials. Using basic criteria necessary to a successful graft (e.g., osteoconduction and osteoinduction), investigators have developed several bone graft substitutes. These can contain a variety of materials, including natural and synthetic polymers, ceramics, and composites; and in some instances, production of bone graft

substitutes can involve biotechnological strategies (i.e., factor- and/or cell-based strategies).

[0006] Osteoinductive substances found in some bone graft substitutes are demineralized bone particles and/or powder. Contained in the extracellular matrix of bone tissue is a full cocktail of bone growth factors, proteins, and other bioactive materials necessary for osteoinduction and, ultimately, successful bone healing. To capitalize on this cocktail of proteins, bone tissue can be demineralized, leaving the osteoinductive agents in the demineralized bone matrix (DBM). Such osteoinductive DBM can be incorporated into a number of different bone graft substitutes.

[0007] While a number of different materials thought to enhance osteoconductivity (i.e., purified or partially purified polymers) have been used in DBM bone graft substitutes; new, more easily prepared, osteoconductive/structural materials for combining with DBM to produce a bone graft substitute are desirable.

SUMMARY OF THE INVENTION

[0008] The present invention provides a biologically functional tissue repair implant having a stable porous sponge-like structure. In one embodiment, the functionally active tissue repair implant of the present invention comprises a uniform composition containing a connective tissue homogenate and a carrier that are evenly distributed to form a three-dimensional framework. The carrier may be a stabilizer of the porous sponge-like structure. The carrier may be a composition containing one or more polysaccharides, polymers, salts, bioactive factors, and/or photoactive agents. The carrier may also be a solution including one or more polysaccharides, polymers, salts, bioactive factors, and/or photoactive agents, and the carrier may be a composition comprising one or more solutes of the carrier solution.

[0009] The tissue repair implant of the present invention may also consist essentially of evenly distributed connective tissue homogenate and carrier in a three-dimensional framework. In another embodiment, the functionally active tissue repair implant comprises a uniform composition further containing bone fragments interspersed in the connective tissue homogenate and carrier framework. The bone fragments are distributed throughout the framework. The tissue repair implant of the present

invention may consist essentially of evenly distributed connective tissue homogenate, carrier, and bone fragments in a three-dimensional framework.

[0010] The tissue repair implant of the present invention may be preshaped, elastic, resilient, and insoluble in water. The implant may maintain its shape in a dry or a wet state. In its dry state, the implant will quickly absorb fluid because it is porous. For example, in its wet state, the implant will maintain its porosity, cohesiveness, and integrity.

[0011] The connective tissue homogenate may contain one or more connective tissues. The bone fragments may be natural or synthetic bone fragments.

[0012] The present invention also discloses methods for producing a biologically functional tissue repair implant having a stable porous sponge-like structure. In one embodiment, the method comprises: (a) producing a connective tissue homogenate from one or more connective tissues; (b) mixing the connective tissue homogenate with a carrier solution to produce a connective tissue carrier; (c) mixing one or more natural or synthetic bone fragments with the connective tissue carrier to produce a tissue repair mixture; (d) freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a framework to entrap the natural or synthetic bone fragments; and (e) treating the frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure. Optionally, a crudely fragmented connective tissue from one or more connective tissues may be mixed with the tissue repair mixture before freezing or freeze-drying.

[0013] In another embodiment, the method for preparing a tissue repair implant having a stable porous sponge-like structure comprises: (a) producing a connective tissue homogenate from one or more connective tissues; (b) mixing the connective tissue homogenate with a carrier solution to produce a tissue repair mixture; (c) freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a framework; and (d) treating the frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure. Optionally, a crudely fragmented connective tissue

from one or more connective tissues may be mixed with the tissue repair mixture before freezing or freeze-drying.

[0014] In another embodiment, the method for preparing a tissue repair implant having a stable porous sponge-like structure comprises: (a) producing a connective tissue homogenate from one or more connective tissues; (b) mixing the connective tissue homogenate with one or more natural or synthetic bone fragments to produce a mixture; (c) adding a carrier solution to the mixture to produce a tissue repair mixture; (d) freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework to entrap the natural or synthetic bone fragments; and (e) treating the frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure.

[0015] Moreover, the steps set forth above for preparing the tissue repair implant may be combined or the sequential order of the steps may be changed. As an example, the carrier solution may be added to the one or more connective tissues for making the connective tissue homogenate. The carrier solution may replace the water that is added to the connective tissues to make the homogenate.

[0016] The stabilized porous sponge-like structure may be treated with a water replacing agent such as glycerol (glycerin USP), adonitol, sorbitol, ribitol, galactitol, D-galactose, 1,3-dihydroxypropanol, ethylene glycol, triethylene glycol, propylene glycol, glucose, sucrose, mannitol, xylitol, meso-erythritol, adipic acid, proline, hydroxyproline, polyethylene glycol, alcohol, or lipids. The stabilized porous sponge-like structure may also be freeze-dried and/or sterilized. Sterilization may be carried out using gamma irradiation, supercritical solution, ethylene oxide, or electronic-beam. Sterilization may be carried out using supercritical fluid. As an example, sterilization may be carried out with supercritical fluid carbon dioxide.

[0017] The methods of the present invention may include combining the tissue repair implant with one or more bioactive supplements. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. The growth or differentiation factors may be a growth factor of the the FGF-family or

TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh, dexamethasone, insulin, transferrin, selenium, ITS, ascorbate, or a combination thereof. The cytokines may be GM-CSF, G-CSF, TNF- α , IL-1 β , IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1 α , MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN- α , or IFN- β . The anti-inflammatory agents may be an IL-1 α R antibody, TNF- α receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF- κ B inhibitors, or inhibitors of MMP. There are various fibroblast growth factors. As an example, the human FGF-family includes 22 members, FGF-1 through FGF-23. (There is no human FGF-15 because FGF-15 is the mouse ortholog of human FGF-19.) Examples of members of the TGF-family may include TGF- α and TGF- β superfamily. The TGF- β superfamily includes TGF- β s (such as TGF- β 1, TGF- β 2, TGF- β 3), activins, inhibins, bone morphogenic factors (BMPs), anti-mullerian hormone (AMH), myostatins, and others.

[0018] BMPs are members of the transforming growth factor (TGF) superfamily, which are a phylogenetically conserved group of proteins. Structurally, the BMPs display extensive conservation among species having seven characteristic cysteine knot domains that participate in the formation of an interchain disulfide bond between monomers to form biologically active dimers. There are 20 isoforms of BMPs. They may be separated into four subfamilies, for example, (1) BMP2 and BMP4; (2) BMP3 and BMP3B (also known as growth/differentiation factor 10 (GDF10)); (3) BMPs 5, 6, 7 and 8; and (4) GDFs 5, 6, and 7.

[0019] The tissue repair implant may also be combined with one or more agents having bioactive supplement binding sites comprising hyaluronan, heparin, heparan sulfate, keratin sulfate, dermatan sulfate, chondroitin sulfate, betaglycan, heparan sulfate proteoglycan, syndecan, biglycan, or decorin. The one or more bioactive supplement binding sites on the agents may increase the affinity of growth factors, differentiation factors, cytokines, or anti-inflammatory agents to said tissue repair implant. Accordingly, the present invention also discloses a tissue repair implant comprising one or more bioactive supplements and one or more agents having bioactive supplement binding sites.

[0020] The methods of the present invention provide a tissue repair implant having a pore size that may be larger than about 50 microns and a void volume that may be from about 10% to about 95% or from about 30 to 80%. The tissue repair implant prepared by the present invention may be in the form of a rod, sheet, cube, tube, particle, sphere, ellipsoid, wedge, or ribbon.

[0021] The connective tissue homogenate used in the methods of the present invention may be made from one more connective tissues of human and/or animal origins. The connective tissue may comprise dermis, fascia, cartilage, tendon, ligament, pericardium, fat, muscle, urethra, small intestine, demineralized bone, non-demineralized bone, or a combination thereof.

[0022] The connective tissue in the connective tissue homogenate used in the methods of the present invention may be cleaned and/or disinfected. The connective tissue may also be devitalized to remove cellular components. The connective tissue may be freeze dried.

[0023] In the methods of the present invention, the connective tissue homogenate may be prepared by homogenizing the connective tissue at temperature from about 15 °C to about 100 °C for a period of time of about 0.5 minutes to about 30 minutes. The homogenization may be carried out in the presence of a solution. The weight percentage of the connective tissue homogenate in the tissue repair implant may be no more than 80% in the dry state, no more than 50% in the dry state, or no more than 20% in the dry state.

[0024] The carrier solution used in the processes of the present invention may comprise one or more polysaccharides selected from the group comprising alginate, propylene glycol alginate, native or crosslinked chitosan, starch, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), xanthan gum, dextran, carrageenan, hyaluronic acid, chondroitin sulfate, locust bean gum, gum tragacanth, gum arabic, curdlan, pullulan, scleroglucan, or lower methoxylpectin. The carrier solution may comprise salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol)

diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC); dimethyl suberimidate (DMS), dimethyl-3-3'-dithiobispropionimidate (DTBP), or acryl azide. The carrier solution may also contain natural and or synthetic polymers such as but limited to native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, MATRIGEL[®], HUMAN EXTRACELLULAR MATRIX[®], proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, polylactic acid, or a combination comprising at least one of the foregoing polymers.

[0025] In one embodiment, the carrier solution used in the methods of the present invention to make the tissue repair implant may comprise bioactive supplements. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. The growth or differentiation factors may include but are not limited to a growth factor of the FGF-family or TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh, dexamethasone, insulin, transferrin, selenium, ITS, ascorbate, or a combination thereof. Examples of cytokines may include GM-CSF, G-CSF, TNF- α , IL-1 β , IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1 α , MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN- α , or IFN- β . The anti-inflammatory agents may include but are not limited to an IL-1 α R antibody, TNF- α receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF- κ B inhibitors, or inhibitors of MMP. There are various fibroblast growth factors. As an example, the human FGF-family includes 22 members, FGF-1 through FGF-23. (There is no human FGF-15 because FGF-15 is the mouse ortholog of human FGF-19.) Examples of members of the TGF-family may include TGF- α and TGF- β superfamily. The TGF- β superfamily includes TGF- β s (such as TGF- β 1, TGF- β 2, TGF- β 3), activins, inhibins, bone morphogenic factors (BMPs), anti-mullerian hormone (AMH), myostatins, and others.

[0026] There are 20 isotypes of BMPs. They may be separated into four subfamilies, for example, (1) BMP2 and BMP4; (2) BMP3 and BMP3B (also known as growth/differentiation factor 10 (GDF10)); (3) BMPs 5, 6, 7 and 8; and (4) GDFs 5, 6, and 7.

[0027] The carrier solution may comprise bioactive supplements extracted from tissue selected from the group comprising demineralized bone matrix, basement membrane, or submucosa matrix. In another embodiment, the carrier solution may also comprise antioxidants selected from the group comprising sodium nitroprusside, cartilage matrix glycoprotein (CMGP), vitamins C, vitamin E, selenium, N-Acetylcysteine (NAC) estradiol, glutathione, melatonin, resveratrol, flavonoid, carotene, aminoguanidine, or lycopene.

[0028] Alternatively, the carrier solution used in the methods of the present invention may comprise photoactive agents selected from the group comprising a xanthene dye, naphthalimide compounds, riboflavin-5-phosphate, N-hydroxypyridine-2-(1H)-thione, N-(20-ethylaminoethyl)-4-amino-1,8-naphthalimide, bis-diazopyruvamide-N,N9-bis(3-diazopyruvoyl)-2,29-(ethylenedioxy)bis-(ethylamine) (DPD); diazopyruvoyl (DAP); methylene blue, erythrosin, phloxime, thionine, methylene green, rose Bengal, acridine orange, xanthine dye, and thioxanthine dyes, ethyl eosin, eosin Y, or a combination comprising at least one of the foregoing photoactive groups. The photoactive agent may be activated using a UV light or a laser. The weight percentage of the carrier in the tissue repair implant may be no more than 20% in the dry state or no more than 5% in the dry state.

[0029] The natural or synthetic bone fragments materials used in the methods of the present invention to prepare the implants may comprise non-demineralized bone, partially demineralized bone, demineralized bone, ceramics, hydroxyapatite, calcium phosphate, calcium sulfate, calcium carbonate, or a combination of two or more of the above. The natural bone fragments may be from a human or an animal. The natural or synthetic bone fragments may be in the form of particles, fibers, rods, or cubes. The particles may have an average particle size of about 125 micron to about 2000 micron or an average particle size of about 250 micron to about 710 micron. The fibers may have an average width of 0.1 to 2 mm and an average width of

between about 0.3 mm and about 2.5 mm. Fibers may be of varying lengths. An average length of the fiber may range from about 0.3 mm to about 100 mm. The rods may have an average width of 0.5 to 3 mm and an average length of about 1 mm to about 100 mm. The cubes may have an average volume of about 0.001 mm^3 to an average volume of about 1000 mm^3 .

[0030] The demineralized bone used in the methods of the present invention may have a residual calcium content of less than 8% or from about 1% to about 4%. The tissue repair implant may include demineralized bone fragments having between about 0.5 wt % and about 4 wt % residual calcium, about 1 wt% and about 4 wt% residual calcium, or about 2 wt% and about 4 wt% residual calcium in certain embodiments.

[0031] The weight percentage of the natural or synthetic bone fragments used in the methods of the present invention to prepare the tissue repair implant is about 0.1% to about 95% in the dry state, or about 10% to about 90% in the dry state.

[0032] The crudely fragmented connective tissue used in the methods of the present invention may be obtained from tissues that are cleaned, disinfected, optionally devitalized to remove cellular components, and optionally freeze-dried. The crudely fragmented connective tissue may be from tissues comprising fascia, cartilage, ligament, tendon, pericardium, muscle, urethra, small intestine, demineralized bone, dermis, partial demineralized bone, or non-demineralized bone.

[0033] The average diameter of the crudely fragmented connective tissue may be from about 0.01 to about 5 mm. The weight percentage of said crudely fragmented connective tissue in said tissue repair implant is about 0.25% to about 95% in the dry state, or about 0.5% to about 90% in the dry state.

[0034] In the process of the present invention, the tissue repair mixture may be placed in a mold before the freezing or freeze-drying step. The tissue repair mixture may be optionally processed under negative hydrostatic pressure before being frozen to increase porosity.

[0035] The treatment solution used in the methods of the present invention may comprise ionic, enzymatic, or chemical crosslinking agents; or photoactive agents. The treatment solution may comprise salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC); dimethyl suberimidate (DMS), dimethyl-3-3'-dithiobispropionimidate (DTBP), or acryl azide. The treatment solution may comprise natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, demineralized bone matrix, MATRIGEL®, HUMAN EXTRACELLULAR MATRIX®, proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, or polylactic acid, or a combination comprising at least one of the foregoing polymers.

[0036] The treatment solution used in the process of the present invention may comprise one or more bioactive supplements. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. The treatment solution may also contain an agent having one or more bioactive supplement binding sites. The treatment solution may comprise antioxidants to protect bioactive components from oxygen-radical-induced damage.

[0037] In the methods of the present invention, one or more treating steps with one or more treatment solutions may be conducted simultaneously or sequentially to stabilize the porous sponge-like structure and further entrap the natural or synthetic bone fragments. The treatment may be carried out for a duration of about 5 minutes to about 16 hours, about 15 minutes to about 120 minutes, or about 30 minutes to about 60 minutes.

[0038] The present invention also provides a process for repairing a defect in a tissue, for example, bone, cartilage, or soft tissue. In one aspect, the process of the present invention comprises implanting the tissue repair implant having a porous sponge-like structure of the present invention into the site of defect in the tissue without rehydration to allow the tissue repair implant to absorb blood or fluid as well as autologous cells in situ. In another aspect, the process includes rehydrating the tissue repair implant having a porous sponge-like structure of the present invention with a rehydrating solution; optionally seeding cells on the tissue repair implant to render the tissue repair implant vital; optionally culture the cell-seeded tissue repair implant before implantation; implanting the tissue repair implant into the defect. The cells seeded on the implant may be vital cells.

[0039] The rehydrating solution may contain blood or bone marrow aspirate, platelet rich plasma, enzymes, bioactive supplements, natural polymers, synthetic polymers, photoactive agents, antioxidants, crosslinking agents, antimicrobial agents, vital cells, one or more agents that have bioactive supplement binding site(s), or a mixture of two or more of the above. The vital cells may be one or more than one type of cells from autologous or allograft bone marrow aspirate; stromal cells from bone marrow, stromal cells from fat, synovium, periosteum, perichondrium, muscle, dermis, umbilical cord blood, and Warton's jelly; or pericytes. The vital cells may be cells from the placenta, amniotic fluid, peripheral blood, and neural tissue. The vital cells may be stem cells that are totipotent, multipotent, pluripotent, and oligopotent. Examples of stem cells include but not limited to embryonic stem cells, adult stem cells, amniotic stem cells, and induced pluripotent stem cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] Figure 1 shows H&E staining of a porous sponge-like structure containing DBM comprised of a framework created by connective tissue homogenate and alginate, crudely fragmented fascia, and DBM particles. The porous sponge-like structure was freeze-dried and treated with CaCl_2 .

[0041] Figure 2 shows the amount of fluid that various types of porous sponge-like structures can absorb.

[0042] **Figure 3** shows the time required to re-hydrate various types of porous sponge-like structures.

[0043] **Figure 4** shows the remaining percentage of dry weight of various types of porous sponge-like structures after 24 hours of collagenase treatment relative to the pre-treatment dry weights.

[0044] **Figure 5** shows cell viability study using alamar blue assay. Three porous sponge-like structures with DBM particles and various alginate content, fluid content, and duration of CaCl_2 treatment were used for the study.

[0045] **Figure 6** shows a representative hematoxylin and eosin (H&E) staining of an explanted DBM porous sponge-like structure demonstrating new bone formation.

[0046] **Figure 7** shows a representative soft x-ray radiograph of an explanted DBM porous sponge-like structure demonstrating new bone formation in an athymic rat calvaria critical size defect.

[0047] **Figure 8** shows a representative soft x-ray radiograph of an explanted DBM porous sponge-like structure demonstrating new bone formation (white arrows) in an athymic rat posterolateral lumbar spine fusion model.

DESCRIPTION OF THE INVENTION

[0048] The invention discloses a biologically functional tissue repair implant having a porous sponge-like structure useful for repairing defects in tissues. The tissues may be bone tissues, cartilage, or soft tissues. Examples of soft tissues may include tendon, ligament, dermis, and rotator cuff.

[0049] The invention also provides processes for making a tissue repair implant having a porous sponge-like structure to repair bone, cartilage, or soft tissue defects by producing a connective tissue homogenate from one or more connective tissues; mixing the connective tissue homogenate with a carrier solution to produce a connective tissue carrier; optionally mixing one or more natural or synthetic bone fragments with said connective tissue carrier to produce a tissue repair mixture;

freezing or freeze-drying the tissue repair mixture to produce a porous sponge-like structure and create a framework to entrap the natural or synthetic bone fragments; treating the frozen or freeze-dried porous sponge-like structure with one or more treatment to produce a stabilized porous sponge-like structure. A crudely fragmented connective tissue from one or more connective tissues is optionally mixed with the tissue repair mixture before freezing or freeze drying. The tissue repair implant having a porous sponge-like structure is optionally combined with one or more bioactive supplement or one or more agents that have bioactive supplement binding site(s) to increase the affinity of growth factors, differentiation factors, cytokines, or anti-inflammatory agents to the tissue repair implant. The invention is further directed toward applying such tissue repair implant for tissue repair.

[0050] The invention also relates to a process for repairing a defect in a human or animal by implanting the tissue repair implant having a porous sponge-like structure into a defect without rehydration to allow the tissue repair implant to absorb blood or fluid as well as autologous cells in situ. Alternatively, implantation of a tissue repair implant into a human or animal can be conducted by re-hydrating the tissue repair implant with a rehydrating solution; optionally seeding vital cells on the tissue repair implant to render the tissue repair implant vital; optionally culture the cell-seeded tissue repair implant before implantation; and implanting the tissue repair implant into the defect.

[0051] Moreover, the invention is directed to producing tissue repair implants having a porous sponge-like structure using a connective tissue homogenate from one or more connective tissues, a carrier solution, and with or without one or more natural or synthetic bone fragments for tissue repair. The invention relates to a process for making a porous sponge-like structure and stabilizing the porous sponge-like structure through one or more freeze drying cycles and one or more treating steps using one or more treatment solutions.

[0052] In the present application, the term "connective tissue" refers to mesodermally and endodermally derived tissue that may be more or less specialized, and that may be, at least in part, made up of fibers. Most of the connective tissues contemplated in the present invention are less specialized tissues that are rich in extracellular matrix

(i.e., collagen, proteoglycan, among others), and that surround other more highly ordered tissues and organs. A relatively, more specialized tissue contemplated in the present invention is cartilage. Varieties of connective tissue that may be used in the present invention include but not limited to adipose; loose connective tissue; dense, regular, irregular, or elastic connective tissue; white fibrous connective tissue; and cartilage. Connective tissue may be classified according to concentration of fibers as loose (areolar) and dense, the latter having more abundant fibers than the former. Connective tissues may be obtained from vertebrates. In some embodiments, the tissues may have human, bovine, equine, porcine, ovine, caprine, or piscine origins, among others. Connective tissues may also be the product of biotechnological methods, for example, tissue engineered connective tissues produced using cell culture methods.

[0053] Specific examples of connective tissues that may be used in certain embodiments of the present invention include but are not limited by at least, fascia, dermis tendons, ligaments, pericardium, urethra, small intestine, muscle, bone, dermis, or cartilage. Examples of different types of fascia that may be used in certain embodiments of the present invention include: fascia lata, fascia adherens, fascia brachii, fascia axillaris, antebrachial fascia, abdominal fascia, internal fascia, fascia iliaca, fascia profunda, clavipectoral fascia, fascia cribosa, crucial fascia, deltoid fascia, dorsal deep fascia, pelvic fascia, fascia cruris, lumbar fascia, and pectoral fascia, among others. "Crudely fragmented connective tissue" refers to connective tissue that has been sliced, ground, carved, chipped, chopped, minced, cut, dissected, rent, ripped, sectioned, snipped, diced, shaved, comminuted, or trimmed into fragments. Such fragmented connective tissue may have an average diameter greater than about 50 microns and less than about 0.5 cm, for example, having cut dimensions of approximately 0.5 x 0.5 cm, and a thickness appropriate to the tissue being crudely fragmented. In some embodiments, the crude fragments may not be of uniform size. "Homogenized connective tissue" or "connective tissue homogenate" contains connective tissue that has been reduced to particles that are uniformly small and evenly distributed. Homogenized connective tissue may optionally include at least one of water, aqueous solutions, or water miscible polar organic solvents, in addition to the particles. The homogenized connective tissues used in methods of the present invention include particles having an average diameter of less than about 50

microns. In some embodiments, the homogenized connective tissue may be prepared by shear-induced shredding of a composition comprising connective tissue, and optionally, at least one of water, an aqueous solution and a water miscible polar organic solvent. A conventional blender may be used in preparing the homogenized connective tissue, in certain embodiments.

[0054] "Natural or synthetic bone" is meant to refer to bone tissue, tissue resembling bone, synthetic material resembling bone, and tissue capable of forming bone. The term "bone" or "bone tissue" is intended for the purposes of the invention to refer to for example, an autograft bone, an allograft bone, or an xenograft bone. Such bone includes any bone from any source, including but not limited to bone from a living human donor, bone from a human cadaveric donor, and bone from an animal. The bone may include cortical bone and/or cancellous bone and/or cortico-cancellous bone. The term "bone fragment," as used in the present application refers to ground bone, pulverized bone, bone cubes, bone chips, bone strips, bone particles, and bone fibers. Bone fragments may be "bone particles" or "bone fibers," in some embodiments of the present invention. "Bone particle" refers to a piece of bone having an average diameter of between about 125 microns and about 4 mm. "Bone fiber" refers to a filament or thread of bone having an average thickness of between about 0.1 mm and about 2 mm and an average width of between about 0.3 mm and about 2.5 mm. Fibers can be of varying lengths. In certain embodiments, a bone fiber can have an average length of between about 1.0 mm and about 100 mm. In certain embodiments, bone fiber contains lamellae in the shape of threads or filaments having a median length to median thickness ratio of about 10:1. Synthetic material resembling bone comprises ceramics, hydroxyapatite, calcium phosphate, or calcium sulfate.

[0055] "Demineralized bone," as used in the present application refers to bone having less than about 8 wt % residual calcium. Demineralization involves treating the surface of a bone tissue to remove a surface layer of its inorganic mineral hydroxyapatite material leaving the mechanical properties of the organic phase of the bone constructs substantially unchanged. The level of demineralization of a bone tissue is defined by the amount (wt %) of residual calcium found in the demineralized bone. In some embodiments, the demineralized bone may contain

physiologically active levels of growth and differentiation factors, for example, bone morphogenetic proteins (BMPs).

[0056] In some embodiments, the homogenized connective tissue may be prepared from allogenic or xenogenic tissue. Such connective tissue may be obtained from a human donor or an animal, such as bovine, porcine, or murine etc. Connective tissue may be obtained relatively economically. Varieties of connective tissue that may be used in certain embodiments of the present invention include but are not limited to areolar or loose connective tissue; adipose; dense, regular or irregular connective tissue; white fibrous; elastic connective tissue; and cartilage. Specific examples of connective tissues that may be used in certain embodiments of the present invention include, for example, fascia, dermis, tendons, ligaments, pericardium, urethra, small intestine, muscle, bone, and cartilage. Examples of different types of fascia that may be used in some embodiments of the present invention include: fascia lata, fascia adherens, fascia brachii, fascia axillaris, antebrachial fascia, abdominal fascia, internal fascia, fascia iliaca, fascia profunda, clavipectoral fascia, fascia cribosa, crucial fascia, deltoid fascia, dorsal deep fascia, pelvic fascia, fascia cruris, lumbar fascia, and pectoral fascia, among others. For practical reasons of availability during procurement and amount of fascia available, fascia lata from the anterior portion of the upper leg may be used in certain embodiments. Homogenized connective tissue may be prepared by methods involving, cleaning and disinfecting connective tissue, and removing extraneous tissues associated with the connective tissue. Connective tissues may be cut into small pieces to produce crudely fragmented connective tissue, and optionally triturated and washed with distilled/deionized endotoxin-free water and/or an aqueous solution, such as isotonic saline, among others. In processing, multiple "washes" may be affected using volumes of aqueous solution that are 10 times the approximated volume of the tissue being processed, in some embodiments. The use of three such processing steps may affect an approximate 1:1000 dilution of associated solubilizable elements rendering the tissue essentially free from such solubilizable elements. Connective tissue may be treated and homogenized at temperatures sufficient to produce a flowable homogenized connective tissue, in certain embodiments. The homogenized connective tissue may include connective tissue that has been reduced to particles that are uniformly small and evenly distributed. Homogenized connective tissue and/or the carrier may optionally

include at least one of water, aqueous solutions, for instance isotonic saline, and water miscible polar organic solvents in addition to the connective tissue particles. The homogenized connective tissues used in methods of the present invention may include particles having an average diameter of less than about 50 microns, less than about 20 microns, or less than about 50 microns and greater than about 5 microns. In some embodiments, the homogenized connective tissue and optionally, at least one of a water miscible polar organic solvent, water and an aqueous solution, may be prepared by shear-induced shredding of connective tissue. A conventional blender may be used in preparing the homogenized connective tissue, in certain embodiments.

[0057] Certain methods for producing the inventive tissue repair implants may include preparing a connective tissue homogenate. Prior to homogenization, connective tissues, such as dermis, fascia, tendons, ligaments, pericardium, muscle, bone, urethra, small intestine, and cartilage, among others, may be crudely fragmented. Connective tissue, which may be fresh or freeze-dried, may be sliced, ground, carved, chipped, chopped, minced, cut, dissected, rent, ripped, sectioned, snapped, diced, shaved, comminuted, or trimmed into crude fragments. In some embodiments, the crude fragments may have an average diameter greater than about 50 microns. The crude fragments may be of varying sizes, in some embodiments. Essentially intact connective tissue or crude fragments of connective tissue, which may be fresh or freeze-dried, may be homogenized at least one time to prepare the homogenate. The homogenization step(s) of certain inventive methods may involve shear-induced shredding of connective tissue. Connective tissue may be homogenized to have tissue fragments having an average diameter of less than about 50 microns, less than about 20 microns, or less than about 50 microns and more than about 5 microns. Water, at least one aqueous solution, such as isotonic saline, or other components may be combined with a connective tissue before homogenization.

[0058] Certain methods include at least one of (a) heating a connective tissue before it is homogenized, (b) heating a connective tissue while it is being homogenized, and (c) heating a connective tissue homogenate. In some embodiments the heating is done to a temperature of between about ambient temperature and about 100 °C, or between about 37 °C and about 100 °C. Heating may be carried out for between

about 0.5 minutes and about 30 minutes. Heating may be accomplished using sonication, microwave irradiation, or conventional heat transfer from a heating component, among other methods known in the art.

[0059] The tissue repair implant of the present invention has a "porous sponge-like structure". The term "porous sponge-like structure" refers to a three-dimensional structure that is porous, coherent, elastic, flexible, fibrous and resilient. In a dry state, the porous sponge-like implant of the present invention can quickly absorb fluid. In the wet state, the porous sponge-like implant of the present invention can maintain the porosity, cohesiveness, and integrity. The wet porous sponge-like structure can resist certain tensile stress, and bounce back and reabsorb fluid after being released from compression. The porous sponge-like implant can be twisted, folded, rolled, and inserted into the defect or wrapped around the defect of bone, cartilage or soft tissue.

[0060] In one embodiment, the present invention provides a process of making a tissue repair implant having a porous sponge-like structure. The process comprising preparing a connective tissue homogenate from one or more connective tissues; mixing the homogenate with a carrier solution to produce a connective tissue carrier; and mixing the connective tissue carrier with one or more natural or synthetic bone fragments to produce a tissue repair mixture. In another embodiment, the present invention provides a process of preparing a tissue repair implant having a porous sponge-like structure comprising obtaining a connective tissue homogenate from one or more connective tissues and mixing the connective tissue homogenate with a carrier solution, which does not contain natural or synthetic bone fragments, to produce a tissue repair mixture. A crudely fragmented connective tissue may be added into the tissue repair mixture to increase the integrity of the porous sponge-like structure. The connective tissue homogenate or the crudely fragmented connective tissue may be derived from one or more connective tissues of human or other animal(s). The natural or synthetic bone fragments may be in the form of particles, fibers, rods or cubes. The natural or synthetic bone fragments may have demineralized bone, partially demineralized bone, non-demineralized bone, ceramics, hydroxyapatite, calcium phosphate, calcium sulfate, calcium carbonate, or a combination of two or more of the above. In one embodiment of the invention, the

mixture can be distributed in a pre-designed mold and kept at a temperature between 0 °C to about 30 °C to allow initial gelation and form a non-flowable mixture. In another embodiment of the invention, in order to maintain the porosity and an even distribution of pores within the porous sponge-like structure, the tissue repair mixture can be frozen first to solidify the structure and then optionally freeze-dried. A three-dimensional framework in the form of a web (Figure 1), created by mixing the connective tissue homogenate with the carrier solution, may be treated with a treatment solution to further stabilize and increase the mechanical strength of the structure.

[0061] Certain embodiments of the present invention are directed to tissue repair implants comprising a plurality of demineralized bone fragments or non-demineralized bone fragment embedded in a porous three-dimensional framework. The framework may include at least one homogenized connective tissue and at least one carrier. In some embodiments, the tissue repair implant may be safely used in repairing damaged tissue, for instance bone in a patient. The tissue repair implant may, in some embodiments, be biocompatible, osteoinductive, and/or osteoconductive, such that it may ultimately be remodeled to a mineralized, hard tissue at the application site in vivo. In certain embodiments, the tissue repair implants may further include at least water, an aqueous solution, or a water miscible polar organic solvent. The tissue repair implant may include materials that improve handling or functional characteristics post-implantation. In some embodiments, the bone and connective tissue may be obtained from the same donor source (i.e., a single human cadaver donor). In certain embodiments, the tissue repair implant may be aseptic or sterile.

[0062] Each of the demineralized bone fragments and/or non-demineralized bone fragments and the homogenized connective tissue of the inventive implant may include materials derived from allogenic or xenogenic sources. In certain embodiments, bone and connective tissues obtained from vertebrate species, for example human, bovine, porcine, ovine, caprine, and piscine sources may be used to prepare demineralized bone fragments and carrier. The plurality of bone fragments may include more than one type of bone tissue (e.g., cancellous, cortical, or cortico-cancellous bone), and the homogenized connective tissue may include more than one

type of connective tissue (i.e., fascia and tendon). The plurality of demineralized bone fragments and/or non-demineralized bone fragments may include bone from a single donor source, or from multiple donor sources, and the homogenized connective tissue may also include tissue from a single donor source, or from multiple donor sources.

[0063] In one embodiment, the water insoluble porous sponge-like implant of the present invention comprises a uniform composition containing evenly distributed connective tissue homogenate and carrier that forms a three-dimensional framework. In another embodiment, the implant of the present invention comprises a uniform composition containing a connective tissue homogenate and carrier framework interspersed with bone fragments. The connective tissue homogenate and the carrier form a uniform framework which traps or embraces the bone fragments.

[0064] The present invention provides an implant having a porous sponge-like structure and is insoluble in water. The implant may include connective tissue, bone, and one or more carriers. The connective tissue may be a homogenized connective tissue, crudely fragmented connective tissue, or a combination of both. Thus, the connective tissue in the implant may be a homogenate, and the implant may comprise homogenized connective tissue and crudely fragmented connective tissue. The bone may be demineralized, non-demineralized, or a combination of both. The bone may be synthetic, naturally occurring, or a combination thereof. The bone may be bone fragments, bone powder, bone fiber, or bone particles. The bone may also be frozen or freeze dried. The carrier may be a solution including one or more polysaccharides such as alginate, one or more polymers, one or more salts, or a combination thereof. The carrier may also be a composition including one or more of the solutes of the carrier composition.

[0065] The following are examples of implants of the present invention. The implant may include homogenized connective tissue and a polysaccharide. The implant may include or consist essentially of homogenized connective tissue and a polysaccharide. The implant may include homogenized connective tissue, a polysaccharide, and demineralized bone. The implant may include homogenized connective tissue, a polysaccharide, and non-demineralized bone. The implant may include homogenized

connective tissue, a polysaccharide, demineralized bone, and non-demineralized bone. The homogenized connective tissue may be fascia. The implant may also include crudely fragmented fascia in addition to homogenized connective tissue. The polysaccharide may be an alginate. The non-demineralized bone may be ground cortical bone. The implant may also include polymers and salts.

[0066] Various factors and agents may be added to the implants of the present invention. In one embodiment, bioactive factors such as growth factors may be added to the implants. In another embodiment, cells may be seeded onto the implants. The implants of the present invention may be used as a vehicle for delivering bioactive factors and vital cells to damaged tissues. Accordingly, the implants of the present invention may be used as a delivery vehicle. As an example, the implants may be used for delivering bioactive factors and vital cells to injured or damaged sites in a subject. The implant of the present invention having homogenized connective tissue and a polysaccharide may act as a non-bone delivery vehicle.

[0067] The connective tissue homogenate may have excellent histocompatibility and elicit minimal antibody formation or immunological rejection, in certain embodiments. The homogenized connective tissue may be made acellular by treating it with detergents, endonucleases and decontaminating agents.

[0068] A devitalized process used to prepare homogenized connective tissue of the present invention may be performed without damage to matrix and/or tissue structure, in some embodiments and may employ detergents, sarcosinates, and decontaminating agents. The matrix structure may include collagens, hyaluronins, elastins, mucopolysaccharides and proteoglycans, among other components. Connective tissue that is devitalized may have a thickness that does not exceed about 8 mm, about 6 mm, or about 4 mm, in certain embodiments. Devitalization processing may be altered to accommodate the thicker tissues.

[0069] In certain aspects, the tissue repair implant may include no more than about 80 wt %, no more than about 50 wt %, or no more than about 20 wt % of the connective tissue homogenate in the dry state. The amount of connective tissue

homogenate added in a tissue repair implant may be varied to adjust the viscosity and gelation characteristics of the tissue repair mixture as well as the re-hydration characteristics of the porous sponge-like structure. Moreover, incorporating connective tissue homogenate in the tissue repair mixture, specifically, in the connective tissue carrier, may strengthen the three-dimensional framework and increase the integrity of the porous sponge-like structure. Incorporating connective tissue homogenate in the tissue repair mixture also may increase the cellular response towards the framework of the porous sponge-like structure by facilitating cellular attachment, migration, and proliferation since the connective tissue homogenate comprises natural or heat-denatured collagen.

[0070] In certain embodiments, the tissue repair implant may include between about 0.25 wt % and about 95 wt %, or between about 0.5 wt % and about 90 wt % of the crudely fragmented connective tissue in the dry state. The amount of crudely fragmented connective tissue added in a tissue repair mixture may be varied to adjust the gelation characteristics and prevent shrinkage of the tissue repair mixture during initial gelation, freeze-drying, and/or one or more treating steps with treatment solution. The crudely fragmented connective tissue may also provide structure reinforcement and further increase the integrity and mechanical strength of the porous sponge-like structure. Moreover, incorporating crudely fragmented connective tissue in the tissue repair implant may further increase the cellular response by facilitating cellular attachment, proliferation, and migration since the crudely fragmented connective tissue comprises collagen.

[0071] In certain embodiments, the tissue repair implant may include demineralized bone fragments having less than about 8 wt % or less than about 4 wt % residual calcium. The tissue repair implant may include demineralized bone fragments having between about 0.5 wt % and about 4 wt % residual calcium, about 1 wt% and about 4 wt% residual calcium, or about 2 wt% and about 4 wt% residual calcium in certain embodiments.

[0072] The plurality of demineralized bone fragments may include at least one of demineralized bone particles and demineralized bone fibers, in some embodiments. The demineralized bone fragments may include materials derived from allogenic or

xenogenic sources. The demineralized bone fragments may be derived from cortical bone or cancellous bone. In certain embodiments, the plurality of demineralized bone fragments may include at least one of demineralized allogenic cortical bone particles, demineralized xenogenic cortical bone particles, demineralized allogenic cancellous bone particles, or demineralized xenogenic cancellous bone particles.

[0073] Certain tissue repair implants of the present invention may include demineralized bone particles. Demineralized bone particles may be prepared from cleaned and disinfected bone fragments that have been freeze-dried and ground/fractured into bone particles. Bone particles may be selected by, for example, using sieving devices (i.e., mesh sieves) commercially available for obtaining particles within a desired size range. Such demineralized bone particles may have an average diameter of between about 125 microns and about 4 mm; between about 710 microns and about 2 mm; between about 125 microns and about 500 microns; between about 125 microns and about 850 microns; between about 125 microns and about 710 microns; or between about 250 microns and about 710 microns. Certain embodiments of the present invention may include demineralized bone particle that is commercially available. For example, a suitable demineralized bone particle that is widely and reliably available is produced by LifeNet Health, Virginia Beach, Virginia.

[0074] Some tissue repair implants of the present invention may include demineralized bone fibers. In certain embodiments, the demineralized bone fibers may have an average thickness of between about 0.1 mm and about 0.3 mm and an average width of between about 0.3 mm and about 1.0 mm. The length of the fibers may vary. In some embodiments, the demineralization process begins by producing bone particles having an average diameter size range of between about 1 mm and about 2 mm or bone fibers having an average dimension of 0.1 mm to 0.3 mm thickness and an average width of about 0.3 mm to about 1 mm. The fragments may then be treated with cleaning solutions. If the bone to be processed into fragments has not been previously cleaned and/or disinfected, they may be cleaned and/or disinfected by the use of detergents, hydrogen peroxides, antibiotics, and/or alcohols to affect a removal of associated tissues such as bone marrow and cellular elements. Following cleaning and disinfection, these fragments (i.e., particles and fibers) may

be demineralized by exposure to dilute hydrochloric acid to affect a removal/reduction of the mineral component of the bone fragments (i.e., particles and fibers). Such additional processing may, in some instances, inactivate potential viral contamination (i.e., HIV and hepatitis viruses, among others).

[0075] Certain embodiments of the present invention are directed to methods for the preparation of inventive tissue repair implant having a porous sponge-like structure, as described above. Such methods include combining a homogenized connective tissue, a carrier solution, a plurality of demineralized or non-demineralized bone fragments, and crudely fragmented connective tissue. The tissue repair implant having a porous sponge-like structure can contain between about 0.1 wt % and about 95 wt % or between about 10 wt % and about 90 wt % demineralized or non-demineralized bone fragments. Bone fragments may include at least one of bone particles and bone fibers from bone tissue. Some methods of preparing the tissue repair implants of the present invention may include the production of particles or fibers from bone tissue, as discussed above. Bone fragments may be demineralized, as described above, in certain embodiments. The fragments may be demineralized to have less than about 8 wt % residual calcium, less than about 4 wt % residual calcium, between about 0.5 wt % and about 4 wt % residual calcium, between about 1 wt% and about 4 wt% residual calcium, or between about 2 wt% and about 4 wt% residual calcium, in some methods of the present invention. Certain methods of the present invention may include freeze-drying demineralized bone fragments. In some embodiments, the demineralized bone fragments may be freeze-dried to a point such that the freeze-dried fragments have an average residual moisture of less than about 10 wt %, or less than about 5 wt %. In some embodiments, freeze-dried demineralized bone fragments may be rehydrated before use in preparing the tissue repair implants having a porous sponge-like structure of the present invention. Rehydrated freeze-dried demineralized bone particles may have a residual moisture content of less than about 80 wt %, less than about 50 wt %, less than about 25 wt %, or between about 10 wt% to about 25 wt %, in certain embodiments.

[0076] In some embodiments of the present invention, the connective tissue carrier will contain a carrier solution in addition to the connective tissue homogenate. Due to the hydrophilic nature of the connective tissue homogenate and the carrier

solution, these two components can penetrate each other and fully integrate so that the connective tissue carrier can form a three-dimensional framework to entrap the natural or synthetic bone fragments. The carrier solution can function as a diluent for the connective tissue homogenate to increase the porosity in the final porous sponge-like structure as well as to change the thickness of the web wall of the three-dimensional framework after one or more freeze-dry cycles. Addition of the carrier solution may change the viscosity of the tissue repair mixture so that the natural or synthetic bone fragments, such as demineralized or non-demineralized bone fragments, will remain "afloat" and evenly distributed in the wet state of tissue repair mixture. The weight percentage of the carrier in the tissue repair implant may be no more than 20% or may be no more than 5% in the dry state.

[0077] The carrier solution may comprise alginate, propylene glycol alginate, native or crosslinked chitosan, starch, polyethylene glycol, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), xanthan gum, dextran, hyaluronic acid, chondroitin sulfate, locust bean gum, gum tragacanth, gum arabic, curdlan, pullulan, scleroglucan, lower methoxylpectin, or carrageenan. The carrier solution may comprise salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC); dimethyl suberimidate (DMS), dimethyl-3-3'-dithiobispropionimidate (DTBP), or acryl azide. The carrier solution may also comprise natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, MATRIGEL[®], HUMAN EXTRACELLULAR MATRIX[®], proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, polylactic acid, or a combination comprising at least one of the foregoing polymers.

[0078] In one embodiment of the present invention, a water replacing agent, such as glycerol (glycerin USP), adonitol, sorbitol, ribitol, galactitol, D-galactose, 1,3-dihydroxypropanol, ethylene glycol, triethylene glycol, propylene glycol, glucose, sucrose, mannitol, xylitol, meso-erythritol, adipic acid, proline, hydroxyproline, polyethylene glycol, alcohol, or lipids, can be included in the carrier solution to increase the flexibility of the porous sponge-like structure in the dry state and act as a moisture barrier. In another embodiment of the present invention, the carrier solution can also include one or more agents that have bioactive supplement binding site(s) so that when the porous sponge-like structure is implanted in vivo or exposed to a biological environment, it can attract cells or bioactive agents from the environment to the porous sponge-like structure to facilitate the healing process. Agents that have one or more bioactive supplement binding sites include but are not limited to hyaluronan, heparin, heparin sulfate, keratin sulfate, dermatan sulfate, chondroitin sulfate, betaglycan, heparan sulfate proteoglycan, syndecan, biglycan, or decorin.

[0079] In one embodiment of the present invention, the carrier solution may also include one or more bioactive supplements to directly stimulate the healing process. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. The growth or differentiation factors may be for example, a growth factor of the FGF-family or TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh (Indian Hedgehog Homolog), dexamethasone, insulin, transferrin, selenium, ITS supplement, ascorbate, or a combination thereof. The cytokines may include GM-CSF, G-CSF, TNF- α , IL-1 β , IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1 α , MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN- α , or IFN- β . Examples of anti-inflammatory agents may include an IL-1 α R antibody, TNF- α receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF- κ B inhibitors, or inhibitors of MMP. There are various fibroblast growth factors. As an example, the human FGF-family includes 22 members, FGF-1 through FGF-23. (There is no human FGF-15 because FGF-15 is the mouse ortholog of human FGF-19.) Examples of members of the TGF-family may include TGF- α and TGF- β superfamily. The TGF- β superfamily includes TGF- β s (such as TGF- β 1, TGF- β 2, TGF- β 3), activins, inhibins, bone morphogenic factors (BMPs), anti-mullerian hormone (AMH), myostatins, and others. There are 20

isotypes of BMPs. They may be separated into four subfamilies, for example, (1) BMP2 and BMP4; (2) BMP3 and BMP3B (also known as growth/differentiation factor 10 (GDF10)); (3) BMPs 5, 6, 7 and 8; and (4) GDFs 5, 6, and 7.

[0080] The carrier solution may include bioactive supplements extracted from tissue comprising demineralized bone matrix, basement membrane, or submucosa matrix. The carrier solution may also include antioxidants, for instance, sodium nitroprusside, cartilage matrix glycoprotein (CMGP), vitamins C, vitamin E, selenium, N-Acetylcysteine (NAC) estradiol, glutathione, melatonin, resveratrol, flavonoid, carotene, aminoguanidine, or lycopene to protect bioactive components from oxygen-radical-induced damage antioxidants.

[0081] In certain methods, the tissue repair implant may be cast in a mold. In some embodiments, a method may further include freeze-drying a cast composition or cross-linking a cast composition using ionic, enzymatic, chemical; or photoactive agents known in the art. In one preferred embodiment, a cast tissue repair implant can be freeze-dried first to obtain the porosity, allow even distribution of pores within the porous sponge-like structure, and at the same time to entrap natural or synthetic bone fragments within the three-dimensional framework created by the connective tissue carrier. In order to stabilize the framework of the porous sponge-like structure, further entrap the natural or synthetic bone fragments, and increase the mechanical strength of the structure, the freeze-dried porous sponge-like structure can be treated with a treatment solution. The treatment solution may comprise ionic, enzymatic, chemical, or photoactive crosslinking agents. Examples of ionic crosslinking agents include but are not limited to metal ions selected from the group consisting of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, and iron. Other ionic crosslinking agents include compounds containing phosphate-bearing groups, or compounds that are sulfates or citrates. Examples of enzymatic crosslinking agents include but are not limited to transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC), dimethyl suberimidate (DMS), or dimethyl-3-3'-dithiobispropionimidate (DTBP). Examples of chemical crosslinking agents include but are not limited to glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol)

diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, or acryl azide.

[0082] Accordingly, the treatment solution may comprise agents such as but not limited to salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC); dimethyl suberimidate (DMS), dimethyl-3-3'-dithiobispropionimide (DTBP), or acryl azide. The treatment solution may also comprise natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, demineralized bone matrix, MATRIGEL[®], HUMAN EXTRACELLULAR MATRIX[®], proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, or polylactic acid, or a combination comprising at least one of the foregoing polymers. The treatment solution may also include bioactive supplements. The bioactive supplements may be growth factors, differentiation factors, cytokines, or anti-inflammatory agents. Examples of growth or differentiation factors may include the FGF-family, TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh, dexamethasone, insulin, transferrin, selenium, ITS, ascorbate, or a combination thereof. Cytokines may include, for example, GM-CSF, G-CSF, TNF- α , IL-1 β , IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1 α , MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN- α , or IFN- β . The anti-inflammatory agents may include but are not limited to an IL-1 α R antibody, TNF- α receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF- κ B inhibitors, or inhibitors of MMP. There are various fibroblast growth factors. As an example, the human FGF-family includes 22 members, FGF-1 through FGF-23. Examples of members of the TGF-family may include TGF- α and TGF- β superfamily. The TGF- β superfamily includes TGF- β s (such as TGF- β 1, TGF- β 2, TGF- β 3), activins,

inhibins, bone morphogenic factors (BMPs), anti-mullerian hormone (AMH), myostatins, and others. There are 20 isotypes of BMPs. They may be separated into four subfamilies, for example, (1) BMP2 and BMP4; (2) BMP3 and BMP3B (also known as growth/differentiation factor 10 (GDF10)); (3) BMPs 5, 6, 7 and 8; and (4) GDFs 5, 6, and 7.

[0083] The treatment solution may also include agents having bioactive supplement binding site(s) comprising hyaluronan, heparin, heparin sulfate, keratin sulfate, dermatan sulfate, chondroitin sulfate, betaglycan, heparan sulfate proteoglycan, syndecan, biglycan, or decorin.

[0084] Since the porous sponge-like structure in the tissue repair implant has been created by freeze-drying before the application of one or more treating steps, the treatment solution can easily penetrate and evenly distribute into the entire porous sponge-like structure, which can significantly shorten the duration of treatment to about one or two hours. Shortening the treatment is vitally important for maintaining the biological function of the bioactive component present in the bone particles, such as BMPs in the demineralized bone matrix, which are prone to be inactivated by hydration. After treating with the treatment solution, the stabilized porous sponge-like structure may be washed with isotonic, hypertonic, or hypotonic solution. Then the porous sponge-like structure can be freeze-dried again and stored dry. In one embodiment of the present invention, a water replacing agent such as glycerol (glycerin USP), adonitol, sorbitol, ribitol, galactitol, D-galactose, 1,3-dihydroxypropanol, ethylene glycol, triethylene glycol, propylene glycol, glucose, sucrose, mannitol, xylitol, meso-erythritol, adipic acid, proline, hydroxyproline, polyethylene glycol, alcohol, or lipids may be applied to increase the flexibility of the porous sponge-like structure in the dry state and act as a moisture barrier. The porous sponge-like structure may be treated with a water replacing agent and then stored wet. The porous sponge-like structure can also be treated with a water replacing agent and then freeze-dried and stored.

[0085] In one embodiment of the present invention, the functionally active tissue repair implant can be combined with one or more bioactive supplements. Examples of bioactive supplements include growth factors, differentiation factors, cytokines,

and anti-inflammatory agents. In another embodiment of the present invention, the tissue repair implant can be combined with one or more agents that have bioactive supplement binding sites through covalent coupling or adsorption to increase the affinity of a bioactive supplement to the tissue repair implant. Agents that have bioactive supplement binding sites may be one or a combination of extracellular matrix proteins. Agents that have one or more bioactive supplement binding sites may be a natural or synthetic molecule comprising hyaluronan, heparin, heparin sulfate, keratin sulfate, dermatan sulfate, chondroitin sulfate, betaglycan, heparan sulfate proteoglycan, syndecan, biglycan, or decorin. Moreover, an extra functional group, such as but not limited to, COOH, NH₂, or OH may be added to the natural or synthetic proteins or peptides to facilitate the coating. The extra functional group may change the hydrophilicity or charge. In one embodiment, one or more bioactive supplements or one or more agent(s) that have bioactive supplement binding sites can be incorporated in one or more treatment solutions. In another embodiment, coating with one or more bioactive supplements or one or more agent(s) that have bioactive supplement binding sites can be conducted after one or more treating steps with the treatment solution. Yet, in another embodiment, coating with one or more bioactive supplements or one or more agent(s) that have bioactive supplement binding sites can be conducted during or right before clinical application.

[0086] In one embodiment, one or more polysaccharides are included in the carrier solution in the tissue repair mixture. The preferred polysaccharide comprises alginate, propylene glycol alginate, native or crosslinked chitosan, starch, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), xanthan gum, dextran, locust bean gum, gum tragacanth, gum arabic, curdlan, pullulan, scleroglucan, lower methoxylpectin, or carrageenan. Due to the hydrophilic nature of the connective tissue homogenate and the polysaccharide solution, these two components can penetrate each other and fully integrate so that the connective tissue carrier can form a three-dimensional framework to entrap solid components such as the natural or synthetic bone fragments. In addition, due to the ionic nature of the polysaccharides, by adjusting the pH of the mixture, it is possible to control the ionic interaction between the carrier solution and the collagen components in the crudely fragmented connective tissue as well as in the connective tissue homogenate so that an enhancement of the gelation occurs in the tissue repair

mixture and a non-flowable mixture can be obtained before freezing or freeze-drying. In one embodiment of the present invention, the tissue repair mixture can be freeze-dried first to produce a three-dimensional framework created by the mixture of connective tissue homogenate and the polysaccharide solution, allow even distribution of pores within the porous sponge-like structure, and at the same time entrap natural or synthetic bone fragments within the framework. The dried polysaccharide(s) present in the porous sponge-like structure may be further treated with covalent and/or ionic-crosslinking agents. An ionic-crosslinking agent may be employed because it is easy to apply and is not likely to cause modification of the bioactive components in the bone particles, such as BMPs in the demineralized bone matrix. In one embodiment, sodium alginate with viscosity from about 20 to about 200 and G/M ratio larger than 1 may be mixed with the connective tissue homogenate and freeze-dried to produce a stable three-dimensional framework of the porous sponge-like structure. The porous sponge like structure may then be treated with a solution of divalent salt such as calcium salt and/or a positively charged solution such as poly-L-lysine or chitosan. The treatment step may be conducted for a period of time of about 5 minutes to about 16 hours, from 15 minutes to about 120 minutes, or between 30 minutes to 60 minutes. Treatment of calcium salt on freeze dried porous sponge-like structure instead of the gel-like tissue repair mixture before freeze drying has multiple advantages. For example, treatment of calcium salt on freeze dried porous sponge-like structure does not limit the diffusion of the calcium salt through out the entire porous sponge-like structure and may produce (a) a stronger and evenly distributed cross-links within the porous sponge-like structure; (b) a porous sponge-like structure that can absorb more fluid in less time; and (c) a larger pore area that can accommodate more natural or synthetic bone fragments within a three-dimensional framework of web. Moreover, treatment of calcium salt on freeze-dried porous sponge-like structure will not crosslink the surface first and form a highly crosslinked surface and an unreacted core (inhomogeneous), which will likely occur if treatment of calcium salt is conducted before freeze-drying. The crosslinked surface can decrease and even inhibit the diffusion of the calcium salt towards the core of the structure. In another embodiment of the present invention, application of the calcium salt not only may crosslink the polysaccharide such as sodium alginate, but also may become a source of mineralization in subsequently induced new bone formation. Moreover, comparing to the framework formed by

alginate only, versus one that incorporates the connective tissue homogenate in the alginate solution, the latter can increase the strength and integrity of the three-dimensional framework and produce a porous sponge-like structure that is stronger, more resilient, and can better entrap bone fragments. The weight percentage of the polysaccharide incorporated in the final dry state of the porous sponge-like structure is less than about 20%, less than about 10%, or less than about 5%.

[0087] Three-dimensional (3-D) macro-porous sponge-like structure in the present invention is designed to provide support for the cells until they are organized into a functioning tissue. After implantation, the architecture of the macro-porous sponge-like structure can control the extent of vascularization and tissue ingrowth. The pores of the porous sponge-like structure in the present invention will be highly interconnected, between about 10 to about 800 μm , or between about 50 to about 300 μm . The pores can occupy about 10 to about 95% of the volume in a dry state. The pore size and volume can be adjusted by adding porogens, application of inert gas, or application of a negative hydrostatic pressure before freeze-drying the tissue repair mixture.

[0088] Methods of the current invention may include sterilization of the functional tissue repair implants having a porous sponge-like structure, components of tissue repair implants, and/or sterilization of packaged tissue repair implants having a porous sponge-like structure. Sterilization may involve the use of ionizing radiation, in some embodiments. In other embodiments, the absorbed dose of ionizing radiation may be between about 8.0 KGy and about 50 KGy, between about 8.0 KGy and about 25 KGy, or between about 8.0 KGy and about 18 KGy. In some embodiments, the sterilizing step may include placing the packaged tissue repair implants having a porous sponge-like structure on dry ice and irradiating the packaged composition. In certain embodiments, sterilization may be performed at a temperature of between about -20 °C. and -50 °C. The implants of the present invention may be sterilized using gamma irradiation, supercritical carbon dioxide, ethylene oxide, or electronic-beam.

[0089] Certain tissue repair implants of the present invention may include other elements. Additional elements may include but are not limited to antibiotics (i.e., penicillin), antiviral agents (i.e., Triton X-100, Nonidet P-40, N-lauroyl sarcosinate, Brij-35, and peroxide generating agents), antitumor agents, analgesics, immunosuppressive agents (i.e., bovine intestinal alkaline phosphatase), permeation enhancers (i.e., fatty acid esters, such as the laurate, myristate and stearate monoesters of polyethylene glycol), or nucleic acids.

[0090] The present invention also provides a method of repairing a damaged tissue such as but not limited to a bone, cartilage, or soft tissue. The method comprises implanting the tissue repair implant at the site of the damaged tissue to promote repair or regeneration of the tissue. The present invention also provides a method of inducing tissue formation comprising providing the tissue repair implant to the site of defect to induce tissue formation or regeneration. The implant may also be a vehicle for delivering needed agents such as bioactive factors and vital cells to injured or damaged tissues.

[0091] In some embodiments, for on-site preparation, the tissue repair implant having a porous sponge-like structure can be applied directly without re-hydration to allow the tissue repair implant to absorb blood or fluid as well as autologous cells in situ. On-site preparation has the advantage that permits the addition of optional components at the discretion of the clinician. Prior to implantation, the freeze-dried tissue repair implant can be re-hydrated with one or a cocktail of bioactive supplements or autologous or allogenic bone marrow aspirate, blood, or platelet rich plasma. Alternatively, the tissue repair implant can be combined with one or more bioactive supplements or one or more agent(s) that has bioactive supplement binding sites through covalent coupling or adsorption prior to the clinical application to increase the affinity of exogenous or endogenous bioactive supplement or cytokine to the porous sponge-like structure.

[0092] In some embodiments, the tissue repair implant may be seeded with vital cells to render the tissue repair implant vital just prior to implantation. Alternatively, the tissue repair implant may be seeded with vital cells and cultured in vitro for certain duration and then implanted. The vital cells may comprise one or more than one type

of cells from autologous or allograft bone marrow aspirate; stromal cells from bone marrow, fat, synovium, periosteum, perichondrium, muscle, dermis, umbilical cord blood, and Warton's jelly; or pericytes.

[0093] Various cells may be seeded on the implants of the present invention. The cells may be derived from autologous or allogeneic sources. The cells may be differentiated cells including chondrocytes, osteoblasts, osteoclasts, endothelial cells, epithelial cells, fibroblasts. Additionally, the cells may be totipotent, pluripotent, multipotent, progenitor, or adult somatic stem cells. The stem cells may be derived from embryos, placenta, bone marrow, adipose tissue, blood vessel, amniotic fluid, synovial fluid, synovial membrane, pericardium, dura, peripheral blood, umbilical blood, menstrual blood, baby teeth, nucleus pulposus, brain, skin, hair follicle, intestinal crypt, neural tissue, muscle. The stem cells may be derived from skeletal muscle, smooth muscle, and cardiac muscle. The stem cells may be derived from genetic reprogramming of mature cells, such as induced pluripotent stem cells (iPSCs). The cells may be an isolated population or a mixture of differentiated cells and/or stem cells. The cells may be applied to the implants with or without culture expansion.

[0094] In some embodiments, the tissue repair implant may be provided in a wet state where a water replacing agent can be present. The tissue repair implant may be sufficiently flexible to allow easy insertion into or wrapping around the defect. The presence of water replacing agent will also allow fast fluid exchange and absorption of the fluid such as blood into the tissue repair implant.

[0095] In some embodiments, the tissue repair implant may be provided in a unitary kit that also include a re-hydration agent, a bioactive agent, a bioactive cocktails, or one or more agents that has bioactive supplement binding sites. The kit may include the implant, one or more types of vital cells, and one or more bioactive factors. The kit may be used to treat or repair damaged or injured tissue.

[0096] In other embodiments, the present invention provides a kit for making the tissue repair implant. The kit may include homogenized connective tissue, one or more bone fragments, a carrier solution, treatment solution and a combination

thereof. The homogenized connective tissue may be fascia. The bone fragments may be demineralized or non-demineralized bone fragments or a combination thereof. The bone fragments may be synthetic or naturally occurring bone fragments or a combination of the two. The carrier solution may comprise a polysaccharide such as alginate.

[0097] In certain embodiments, the tissue repair implant may be provided together with other scaffolds or devices, such as a surgical suture, a decellularized or non-decellularized tissue, a synthetic polymer or metallic cage, for treating or repairing injured soft tissues, osseous defects, or spinal injuries. Examples of soft tissue injuries include rotator cuff tears and injuries and ligament tears and injuries. As an example, the implants of the present invention may be combined with dermis to repair or treat rotator-cuff tears or injuries. As an example, the implant of the present invention may also be used in spinal fusion procedures to immobilize vertebrae in subjects in need thereof. The procedure eliminates pain caused by the abnormal motion of vertebrae in a subject. The implants may be used with a graft such as a bone allograft to immobilize vertebrae in subjects in need thereof.

[0098] The tissue repair implants of the present invention may also be used to treat or repair ligament tears or injuries. The implants may be used to treat or repair achilles tendon and anterior cruciate ligament injuries and tears. For example, the implant may be in the form of a sheet and wrapped around a ligament to promote repair of tears or injuries.

[0099] The present invention provides methods of promoting bone repair or regeneration involving applying the tissue repair implant of the present invention to a damaged bone tissue to promote repair or regeneration of the bone tissue. The implant having bioactive factors and/or vital cells may promote repair or regeneration of bone tissue. Some embodiments of the present invention are directed to methods of inducing bone formation involving applying an implant of the present invention to an implant site to induce bone formation.

[0100] The present invention also provides methods for repairing injured or damaged tissues. The method comprises applying the tissue repair implant of the present

invention to a damaged or injured tissue and allowing the implant to promote repair or regeneration of the damaged or injured tissue.

[0101] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the claimed invention. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1. Preparation of tissue repair implants: porous sponge-like structure with DMB particles.

[0102] Fascia lata and bone from a human cadaver were procured and returned to the processing facility under sterile conditions. Donor histories, personal and medical, were obtained following accepted standards of the American Association of Tissue Banks. Microbiological tests were performed following FDA guidelines for testing sterility of products.

[0103] The bone and fascia were cleaned of unwanted tissues and freeze-dried. The freeze-dried fascia was cut into small (about 1.5 cm by 1.5 cm) pieces (e.g., crude fragments). Sterile water in a volume (1 cm³ of isotonic saline corresponds to about 1 g) approximating 20 times the weight of tissue, was added to a container containing the cut fascia. The ingredients were heated to a temperature of 100 degree C using a hot plate, and maintained at this temperature for about 5 minutes. Optionally, water was added to the heated composition to replace the liquid lost to evaporation. The heated composition was transferred into a conventional blender and mechanically homogenized (e.g., blended) for about 1 to 3 minutes. The homogenized connective tissue was re-heated for an additional 3 minutes on a hot plate, and mechanical homogenization was repeated for an additional 4 minutes (e.g., until the mixture was liquefied and homogeneous).

[0104] Ground demineralized bone powder was prepared by impact fragmentation of bone, followed by freeze-drying. The freeze-dried particles were sized using mesh

sieves. Ground demineralized bone particles having a size in the range of about 250 to 710 microns and demineralized to an average weight percent residual calcium of $2 \pm 1\%$ were used.

[0105] Crudely fragmented fascia was made by blending them in a small blender until the fascia fiber diameter is less than 2 mm. Alginate solution was prepared by dissolving alginate powder (Protanal, LF 10/60, FMC Biopolymer, Dramen, Norway) in sterile water to a final concentration of 0.5 to 2%. The solution was filter sterilized and stored at 2°C - 10°C until use. CaCl₂ solution was made by dissolving CaCl₂ powder (FW=147.02, Mallinckrodt, Hazelwood, MO) in sterile water to a final concentration of 100 mM. The solution was filter sterilized and stored at ambient temperature (16°C – 30°C) until use.

[0106] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier that was mixed further with crudely fragmented fascia and sized, freeze-dried demineralized bone powder. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried for 48 to 72 hours, treated with CaCl₂ for 0.25, 0.5, 1, or 2 hours, washed with water, and freeze-dried again for 48 to 96 hours. Before the first freeze-drying cycle, some samples were exposed to a negative hydrostatic pressure to allow the expansion of the DBM mixture to a preset thickness. The resultant porous DBM structure was then sent out for sterilization by gamma irradiation.

[0107] In one of the tissue repair implants, the porous DBM structure contained about 91% DBM particle, 3% alginate, 5% homogenized fascia, and 1% fascia fiber by dry weight. In another tissue repair implant, the porous DBM structure contained about 70% DBM particle, 3% alginate, 12% homogenized fascia, and 15% fascia fiber by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 2. Preparation of tissue repair implants: porous sponge-like structure without bone fragment.

[0108] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, and CaCl_2 solution were prepared as described in Example 1.

[0109] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried, treated with CaCl_2 , washed, and freeze-dried again. Before the first freeze-drying cycle, some samples were exposed to a negative hydrostatic pressure to allow the expansion of the mixture to a preset thickness. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0110] In one of the tissue repair implants, the porous sponge-like structure contained about 20% alginate, 60% homogenized fascia, and 20% fascia fiber by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 3. Preparation of tissue repair implants: porous sponge-like structure with DBM particles and DMB fiber bone.

[0111] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, demineralized bone powder, and CaCl_2 solution were prepared as described in Example 1.

[0112] Demineralized fiber bone was prepared by cutting cortical bone to produce cut fiber bone having an average length from about 1 mm to about 100 mm. Then the cut fiber bone was cleaned and disinfected, and a total of 463 grams of bone materials were demineralized to 2.5% residual calcium using 2 cycles of 0.5 N HCl and acid volumes of 4.0 liters/cycle and 3.0 liters/cycle, 1 cycle of ultrapure water of 3.0 liters/cycle, and 2 cycles ultrapure water plus buffer of 3.0 liters/cycle to terminate the demineralization process. The bone fibers were finally washed in 3.0 liters of ultrapure water, stored frozen at -80 degree C in a sterile container, and freeze-dried for 96 hrs.

[0113] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier, which was mixed further with demineralized fiber bone and sized, freeze-dried demineralized bone powder. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried, treated with CaCl_2 , washed with water, and freeze-dried again. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation. Before the first freeze dry cycle, some samples were exposed to a negative hydrostatic pressure to allow the expansion of the mixture to a preset thickness. In one of the tissue repair implants, the porous sponge-like structure contains about 70% DBM particle, 17% demineralized fiber bone, 3 % alginate, and 10% homogenized fascia by dry weight. In another tissue repair implant, the porous sponge-like structure contains about 56% DBM particle, 35% demineralized fiber bone, 2.5 % alginate, and 6.5% homogenized fascia by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 4. Preparation of tissue repair implants: porous sponge-like structure with DBM fiber bone.

[0114] The homogenized fascia, sodium alginate solution, demineralized fiber bone, and CaCl_2 solution were prepared as described in Example 1 and Example 3.

[0115] To prepare a tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier, which was mixed further with demineralized fiber bone. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried, treated with CaCl_2 , washed with water, and freeze-dried again. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0116] In one of the tissue repair implants, the porous sponge-like structure contained about 87% demineralized fiber bone, 3 % alginate, and 10% homogenized fascia by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 5. Preparation of tissue repair implants: flexible porous sponge-like structure with DBM particles.

[0117] The homogenized fascia, sodium alginate solution, demineralized bone powder, and CaCl_2 solution were prepared as described in Example 1.

[0118] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution first to produce a connective tissue carrier, which was mixed further with sized, freeze-dried demineralized bone powder. After mixing all the components, the mixture was freeze-dried, treated with CaCl_2 , washed with water. Then the porous sponge-like structure was soaked in about 99.5% glycerol solution for about 1 hr. The excessive glycerol solution was removed and the porous sponge-like structure was freeze-dried again. The resultant flexible porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0119] In one of the tissue repair implants, the porous sponge-like structure contained about 26% DBM particle, 1 % alginate, 3 % homogenized fascia, and 70% of glycerol by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 6. Preparation of tissue repair implants: porous sponge-like structure with DBM particles and high or low dose of heparin.

[0120] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, demineralized bone powder, and CaCl_2 solution were prepared as described in Example 1. Heparin stock solution was prepared by dissolving heparin sodium salt grade I-A from porcine intestinal mucosa (Sigma, St. Louis, MO) in sterile water to a final concentration of 10 mg/mL. The solution was filter sterilized and stored at $2^\circ\text{C} - 10^\circ\text{C}$ until use.

[0121] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution, and various amount of heparin solution to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia and the sized, freeze-dried demineralized bone powder. After mixing all the

components, the mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried, treated with CaCl_2 , washed with water, and freeze-dried again for. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0122] In one of the tissue repair implants, the porous sponge-like structure contained about 70% DBM particle, 2.9 % alginate, 12% homogenized fascia, and 15% fascia fiber, and 0.1% heparin by dry weight. In another tissue repair implants, the porous sponge-like structure contained about 70% DBM particle, 2.6 % alginate, 12% homogenized fascia, and 15% fascia fiber, and 0.4% heparin by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 7. Preparation of tissue repair implants: porous sponge-like structure with ground cortical bone particles.

[0123] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, and CaCl_2 solution were prepared as described in Example 1.

[0124] Ground cortical (GC) bone powder was prepared by impact fragmentation of bone, followed by freeze-drying. The freeze-dried particles were sized using mesh sieves. Ground cortical bone particles having a size in the range of about 250 to 1000 microns were used.

[0125] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia, and the sized, freeze-dried ground cortical bone powder. After mixing all the components, the mixture was freeze-dried, treated with CaCl_2 , washed with water, and freeze-dried again. The porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0126] In one of the tissue repair implants, the porous sponge-like structure contained about 85% GC particles, 3 % alginate, 6% homogenized fascia, and 6% fascia fiber

by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 8. Preparation of tissue repair implants: porous sponge-like structure with GC particles crosslinked with genipin.

[0127] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, and ground cortical bone powder were prepared as described in Example 1 and Example 7. Genipin stock solution was made by dissolving genipin (MW= 226.2, Sigma) in 40% molecular grade ethanol to a final concentration of 100 mg/ml. The solution was filter sterilized and used right away.

[0128] To prepare the tissue repair implant, the homogenized fascia was mixed with various amounts of genipin solution to produce a connective tissue carrier. The wet weight percentage of genipin was 1%, 0.5%, or 0.25% in the tissue repair mixture. Then the connective tissue carrier was mixed further with crudely fragmented fascia and the sized, freeze-dried ground cortical bone powder. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes and kept in dark for 4 to 16 hours at room temperature to allow crosslinking by genipin to occur. The structure turned dark blue after crosslinking. Then the crosslinked and molded structure was washed to remove unbound genipin and freeze-dried. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation. The freeze-dried, molded, tissue repair implants with 1% genipin in the wet mixture demonstrated higher mechanical strength than the ones with 0.5 or 0.25 % genipin in the wet mixture.

Example 9. Preparation of tissue repair implants: porous sponge-like structure with GC particles crosslinked with genipin and stabilized with CaCl₂.

[0129] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, ground cortical bone powder, CaCl₂ solution, and genipin were prepared as described in Example 1 and Example 8.

[0130] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution and 1% genipin solution to produce a connective tissue carrier. Then the connective tissue carrier was mixed further with crudely fragmented fascia and the ground cortical bone powder. After mixing all the components, the mixture was distributed into molds with pre-determined shapes and sizes and kept in the dark for 4 to 16 hours at room temperature to allow crosslinking by genipin to occur. The structure turned dark blue after crosslinking. Then the crosslinked and molded structure was washed to remove unbound genipin, freeze-dried, treated with CaCl_2 , washed with water, and freeze-dried again. The resultant porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0131] In one of the tissue repair implants, the porous sponge-like structure contained about 75% ground cortical particle, 2 % alginate, 13% homogenized fascia, and 10% fascia fiber by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

Example 10. Preparation of tissue repair implants: porous sponge-like structure with GC particles absorbed with BMP-2.

[0132] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, ground cortical bone powder, and CaCl_2 solution were prepared as described in Example 1 and Example 7. Sterile recombinant human BMP-2 (rh BMP-2) produced by expressing BMP-2 gene in *E. coli* (355-BEC, R&D systems) was reconstituted in sterile 4 mM HCl containing 0.2% bovine serum albumin to a stock concentration of 0.2 $\mu\text{g}/\mu\text{L}$. The aliquots of rhBMP2 were stored at -10°C to -30°C until use.

[0133] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia and the sized, freeze-dried ground cortical bone powder. After mixing all the components, the mixture was freeze-dried, treated with CaCl_2 , washed water, and freeze-dried again. The porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0134] In one of the tissue repair implants, the porous sponge-like structure contained about 70% GC particles, 3 % alginate, 11% homogenized fascia, and 16% fascia fiber by dry weight. The rh BMP-2 stock solution was added onto the freeze-dried and molded porous sponge-like structure and allowed to absorb for about 0.5 to 2 hours at room temperature. The amount of rh-BMP-2 that was absorbed on the porous sponge-like structure was about 1 micro gram to about 500 microgram per milligram of dry porous sponge-like structure. Then the structure was freeze dried again.

Example 11. Preparation of tissue repair implants: porous sponge-like structure with GC absorbed with DBM digest.

[0135] The homogenized fascia, sodium alginate solution, crudely fragmented fascia, ground cortical bone powder, and CaCl_2 solution were prepared as described in Example 1 and Example 7. The collagenase digested DBM was prepared by digesting weighed DBM with 0.5 to 1 mg/mL collagenase. The digested DBM was centrifuged and the supernatant of the digested DBM was transferred to a new sterile tube and freeze-dried. The freeze-dried DBM collagenase digest was stored at -10°C to -30°C until use.

[0136] To prepare the tissue repair implant, the homogenized fascia was mixed with a sodium alginate solution to produce a connective tissue carrier, which was mixed further with crudely fragmented fascia, and the sized, freeze-dried ground cortical bone powder. After mixing all the components, the mixture was freeze-dried, treated with CaCl_2 , washed with water, and freeze-dried again. The porous sponge-like structure was then sent out for sterilization by gamma irradiation.

[0137] In one of the tissue repair implants, the porous sponge-like structure contained about 70% GC particles, 3 % alginate, 11% homogenized fascia, and 16% fascia fiber by dry weight. The lyophilized collagenase digested DBM was reconstituted with sterile normal saline, added onto the freeze-dried and molded porous sponge-like structure, and allowed to absorb for about 0.5 to 6 hours at room temperature. The amount of DBM that was absorbed on the porous sponge-like structure was

about 0.1 to 100 milligram on about one milligram of dry porous sponge-like structure. Then the structure was freeze dried again.

Example 12. Characterization of the porous sponge-like structure.

[0138] Tissue repair implants having a porous sponge-like structures were prepared according to the processing steps described in Example 1, Example 2, and Example 3.

[0139] Porosity and DBM distribution in the porous sponge-like structure: Various prototypes of tissue repair implants having a porous sponge-like structures containing DBM were analyzed. H&E staining of one of the representative porous sponge-like structure was shown in Figure 1. It was found the framework of the porous sponge-like structure created by the connective tissue carrier (mixture of connective tissue homogenate and alginate, purple color) form web structures with large pores. The average pore size is larger than 100 μm . DBM particles and crudely fragmented fascia were distributed within the framework.

[0140] Hydration experiment: Each porous sponge-like structure containing DBM was cut and the dry weight of each sample was determined. Then, the sample was placed in a plastic weigh boat and 1 mL of 0.9% saline was added to each sample. The time needed for hydration was measured using a timer until the porous sponge-like structure became completely saturated and visibly changed color from white to beige. The hydrated sample was blotted lightly on a paper towel, and the wet weight was determined for each sample.

[0141] Regardless of the application of negative hydrostatic pressure before freeze-drying, the porous sponge-like structures without DBM were able to absorb more fluid than the porous sponge-like structure containing DBM (Figure 2). However, the amount and the type of DBM (particle or fiber) entrapped in the porous sponge-like structure did not change the amount of fluid that the porous sponge-like structure can absorb. The porous sponge-like structure containing DBM can absorb the amount of fluid that is about 80% of its wet weight or about four times of its dry weight. In addition, the application of negative hydrostatic pressure before freeze-

drying tends to decrease the time required to rehydrate the porous sponge-like structure especially in the porous DBM structure containing 69% of DBM particles (Figure 3).

[0142] Collagenase degradation assay: Three replicates of each porous sponge-like structure were weighed and placed in microcentrifuge tubes. For the samples that contained DBM, 30 mg of the porous sponge-like structure were digested with 600 micro-liters of 0.5 mg/mL collagenase in DMEM supplemented with 1% FBS. Only 10 mg of porous sponge-like structures containing no DBM were used for the collagenase degradation assay. All of the degradation samples were digested at 37° C for 16 hours without shaking and an additional 8 hrs with shaking at 1000 r.p.m. The supernatant was separated from the pellet by centrifuging at 10000 g for 10 minutes. The pellets were freeze dried in microcentrifuge tubes, and the dry weight of each pellet was determined. Some porous sponge-like structures containing DBM that were not treated with calcium chloride were used as controls.

[0143] Over 50% (dry weight) of the porous sponge-like structure without DBM was digested, while over 80% of the porous sponge-like structures containing DBM were digested after 24 hrs (Figure 4). Neither the calcium chloride treatment nor the application of negative pressure before freeze-drying changed the degradation. The amount of digested porous sponge-like structure was not different for different amount or type of DBM present within the porous sponge-like structure. Therefore, the encapsulation of the DBM in the porous sponge-like structure did not change the accessibility of the DBM.

Example 13. In vitro biocompatibility

[0144] Mouse fibroblast L929 cells were seeded directly on the surface of the porous sponge-like structure at the density of 0.1×10^6 cells/cm² and cultured. Cells seeded on the tissue culture plate were used as a control. Same seeding density was maintained for the porous sponge-like structure group and the control group. AlamarBlue reagent (10%) was added into the culture media on day 3 of the culture and incubated for 20 hrs. The fluorescent intensity of the culture media with AlamarBlue reagent was measured. The intensity of the fluorescence is

correspondent to the viability/metabolism of the cells, i.e., the higher the intensity the more viable/metabolic active cells. As shown in Figure 5, regardless of the concentration of alginate or the fluid content or CaCl_2 treatment duration, all the porous sponge-like structure tested not only maintained the cell viability but also support cell growth as the cell number in the porous sponge-like structure group was higher than that in the control groups after three day of culture. In addition, the porous sponge-like structure maintained their integrity during the culture.

Example 14. Determination of new bone formation.

[0145] Tissue repair implants having a porous DBM structures were prepared according to the processing steps described in Example 1.

[0146] The prepared tissue repair implants and demineralized bone matrix (DBM) controls (without homogenized connective tissue or carrier) were implanted heterotopically (e.g., into muscle pouches) in the hind quarters of athymic (e.g., nude) mice.

[0147] The amounts of materials implanted were varied to always implant 20 mg of DBM. For example, 30 mg of the tissue repair implant that contains about 69% DBM composition was implanted to reach about 20 mg of DBM. Two to three mice with two implants per mouse were used for each of the tissue repair implant groups and DBM control group.

[0148] After 28 days, the implants were explanted, and one explant from each mouse was fixed. At least one histological section was cut from the center of each of these explants. Samples were fixed in 10% buffered formalin. Standard dehydration, embedding and sectioning protocols were used to produce light microscopy slides that were subsequently stained with hematoxylin and eosin. Using histomorphometric analysis, the percent new bone formed was calculated as a cross-sectional area of newly formed bone (mm^2) divided by the total cross-sectional area (mm^2) for a representative microscopic view of a histology slide multiplied by 100. Every other field of view with at least 50% bone content was used as a representative view with about 10 representative views being analyzed per slide.

[0149] A representative H&E staining of an explanted porous sponge-like structure containing DBM showed new bone formation (Figure 6). All the experimental groups and the controls had at least 9% new bone formation.

Example 15. Assessment of new bone formation in an athymic rat calvaria critical size defect model.

[0150] Tissue repair implants having a porous DBM structures were prepared according to the processing steps described in Example 1.

[0151] Athymic rats were anesthetized with intraperitoneal injection of xylazine (10 mg/kg) and ketamine (50 mg/kg). Using aseptic surgical techniques, an anteroposterior midline skin incision (4-5cm) was made over the cranial vault. The periosteum was elevated and retracted to expose the cranial bone. An 8-mm diameter circular opening was created in the skull of rat using a drill bit. Prior to implantation, each DBM sponge implant was rehydrated. Following implantation of DBM sponge into defect, the skin wound was closed with suture. After recovery from anesthesia, animals were returned to their respective housing area. Six weeks after implantation, rats were euthanized by carbon dioxide asphyxiation and calvarial bones were harvested and fixed in 10% neutral buffered formalin. Soft x-ray was performed for assessment of new bone formation. A representative radiograph of an explant showed new bone formation in the defect (Figure 7, white arrow).

Example 16. Assessment of new bone formation in an athymic rat posterolateral lumbar spine fusion model.

[0152] Tissue repair implants having a porous DBM structures were prepared according to the processing steps described in Example 1.

[0153] Athymic rats were anesthetized with intraperitoneal injection of xylazine (10 mg/kg) and ketamine (50 mg/kg). The posterior spine was approached through a single midline skin incision. The paraspinal muscles were reflected using a scalpel and dissector to expose the spinous and transverse processes of lumbar segments 4-5

(L4 and L5). An electric burr was used to decorticate the transverse processes and exposed cancellous bone. DBM sponge was implanted to each lateral gutter. Six weeks after implantation, rats were sacrificed and new bone formation was demonstrated by radiography (Figure 8, white arrows).

Example 17. Preparation of Tissue Repair Implants: Porous Sponge-Like Structure with DBM and Ground Cortical (GC) Bone Fragment.

[0154] The homogenized fascia, sodium alginate solution, DBM, and ground cortical (GC) bone powder were prepared as described in Example 1 and Example 7.

[0155] To prepare the tissue repair implant, the homogenized fascia was mixed with sized, freeze-dried demineralized bone powder, and GC powder to produce a mixture. Then a sodium alginate solution was added and mixed further to produce a tissue repair mixture. The mixture was distributed into molds with pre-determined shapes and sizes, freeze-dried for 48 to 72 hours, treated with CaCl_2 for 0.25, 0.5, 1, or 2 hours, washed with water, and freeze-dried again for 48 to 96 hours. Before the first freeze-drying cycle, some samples were exposed to a negative hydrostatic pressure to allow the expansion of the DBM mixture to a preset thickness. The resultant porous DBM/GC structure was then sent out for sterilization by gamma irradiation.

[0156] In one of the tissue repair implants, the porous DBM/GC structure contained about 60% DBM particle, 30% GC particles, 1% alginate, and 9% homogenized fascia by dry weight. In another tissue repair implant, the porous DBM/GC structure contained about 75% DBM particle, 10% GC particles, 3% alginate, 12% homogenized fascia by dry weight. The freeze-dried, molded, tissue repair implants demonstrated high mechanical strength and maintained the shape of their mold.

CLAIMS:

Claim 1: A biologically functional tissue repair implant having a porous sponge-like structure comprising a connective tissue homogenate and a carrier, wherein the implant is insoluble in water.

Claim 2: The tissue repair implant of claim 1, further comprising one or more natural or synthetic bone fragments.

Claim 3: A process for preparing a biologically functional tissue repair implant having a porous sponge-like structure comprising:

- a. producing a connective tissue homogenate from one or more connective tissues;
- b. mixing said connective tissue homogenate with a carrier solution to produce a connective tissue carrier;
- c. mixing one or more natural or synthetic bone fragments with said connective tissue carrier to produce a tissue repair mixture;
- d. freezing or freeze-drying said tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework to entrap said natural or synthetic bone fragments; and
- e. treating said frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure.

Claim 4: The process of claim 3, wherein a crudely fragmented connective tissue from one or more connective tissues is mixed with said tissue repair mixture before freezing or freeze-drying.

Claim 5: A process for preparing a biologically functional tissue repair implant having a porous sponge-like structure comprising:

- a. producing a connective tissue homogenate from one or more connective tissues;
- b. mixing said connective tissue homogenate with a carrier solution to produce a tissue repair mixture;

- c. freezing or freeze-drying said tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework; and
- d. treating said frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure.

Claim 6: The process of claim 5, wherein a crudely fragmented connective tissue from one or more connective tissues is mixed with said tissue repair mixture before freezing or freeze-drying.

Claim 7: The process of claim 3, wherein said stabilized porous sponge-like structure is treated with a water replacing agent.

Claim 8: The process of claim 3, wherein said stabilized porous sponge-like structure is freeze-dried.

Claim 9: The process of claim 3, wherein said stabilized porous sponge-like structure is sterilized.

Claim 10: The process of claim 3, wherein said framework of said porous sponge-like structure is created by said connective tissue carrier.

Claim 11: The process of claim 3, wherein said tissue repair implant is combined with one or more bioactive supplements .

Claim 12: The process of claim 3, wherein said tissue repair implant is combined with one or more agents that have bioactive supplement binding site(s).

Claim 13: The process of claim 12, wherein the bioactive supplement binding site(s) increases the affinity of growth factors, differentiation factors, cytokines, or anti-inflammatory agents to said tissue repair implant.

Claim 14: A method of repairing defects in bone, cartilage, or soft tissue comprising implanting the tissue repair implant of claim 3 at the site of defect.

Claim 15: A tissue repair implant having a porous sponge-like structure made according to claim 3, wherein the pore size is larger than about 50 microns.

Claim 16: A tissue repair implant having a porous sponge-like structure made according to claim 3, wherein the void volume is from about 10% to about 95%.

Claim 17: A tissue repair implant having a porous sponge-like structure made according to claim 3, wherein the void volume is from about 30% to about 80%.

Claim 18: The process of claim 3, wherein said tissue repair implant having a porous sponge-like structure is in the form of rod, sheet, cube, tube, particle, sphere, ellipsoid, wedge, or ribbon.

Claim 19: The process of claim 3, wherein said connective tissue homogenate is made from one more connective tissue(s) of human and/or animal origins.

Claim 20: The process of claim 3, wherein said connective tissue comprises fascia, cartilage, tendon, ligament, pericardium, fat, muscle, urethra, small intestine, dermis, demineralized bone, non- demineralized bone or a mixture of two or more of the above.

Claim 21: The process of claim 3, wherein said connective tissue in the connective tissue homogenate is cleaned and disinfected.

Claim 22: The process of claim 20, wherein said connective tissue is devitalized to remove cellular components.

Claim 23: The process of claim 21, wherein said connective tissue is freeze-dried.

Claim 24: The process of claim 3, wherein producing said connective tissue homogenate is carried out by homogenizing said connective tissue at temperature from about 15 °C to about 100 °C for a period of time of about 0.5 minutes to about 30 minutes.

Claim 25: The process of claim 24, wherein said homogenizing is carried out in the presence of a solution.

Claim 26: The process of claim 3, wherein the weight percentage of said connective tissue homogenate in said tissue repair implant is no more than 80% in the dry state.

Claim 27: The process of claim 3, wherein the weight percentage of said connective tissue homogenate in said tissue repair implant is no more than 50% in the dry state.

Claim 28: The process of claim 3, wherein the weight percentage of said connective tissue homogenate in said tissue repair implant is no more than 20% in the dry state.

Claim 29: The process of claim 3, wherein said carrier solution comprises one or more polysaccharides selected from the group comprising alginate, propylene glycol alginate, native or crosslinked chitosan, starch, cellulose and its derivatives (such as cellulose acetate, carboxymethyl cellulose, and methyl cellulose), xanthan gum, dextran, carrageenan, hyaluronic acid, chondroitin sulfate, locust bean gum, gum tragacanth, gum arabic, curdlan, pullulan, scleroglucan, or lower methoxylpectin.

Claim 30: The process of claim 3, wherein said carrier solution comprises salts of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron; glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, transglutaminase, ethylenediamine, lysyl oxidase family,

hexamethylene diisocyanate (HMDIC); dimethyl suberimide (DMS), dimethyl-3-3'-dithiobispropionimide (DTBP), or acryl azide.

Claim 31: The process of claim 3, wherein said carrier solution comprises natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, MATRIGEL[®], HUMAN EXTRACELLULAR MATRIX[®], proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, polylactic acid, or a combination comprising at least one of the foregoing polymers.

Claim 32: The process of claim 3, wherein said carrier solution comprises bioactive supplements selected from the group comprising a growth or differentiation factor of the FGF family, TGF-family, IGF-1, PDGF, EGF, VEGF, HGF, PTHrP, Ihh, dexamethasone, insulin, transferrin, selenium, ITS, or ascorbate.

Claim 33: The process of claim 3, wherein said carrier solution comprises bioactive supplements selected from the group comprising cytokine of GM-CSF, G-CSF, TNF- α , IL-1 β , IL-4, IL-6, IL-8, IL-10, SLP1, MCP1, MIP-1 α , MIP-2, IL-18, angiopoietin, KGF, endothelin, IFN- α , or IFN- β .

Claim 34: The process of claim 3, wherein said carrier solution comprises bioactive supplements selected from the group comprising anti-inflammatory agent of an IL-1 α R antibody, TNF- α receptor antagonist, cyclooxygenase-2 specific inhibitors, MAP kinase inhibitors, NO synthase inhibitors, NF- κ B inhibitors, or inhibitors of MMP.

Claim 35: The process of claim 3, wherein said carrier solution comprises bioactive supplements extracted from tissue selected from the group comprising demineralized bone matrix, basement membrane, or submucosa matrix.

Claim 36: The process of claim 3, wherein said carrier solution comprises antioxidants selected from the group comprising sodium nitroprusside, cartilage matrix glycoprotein (CMGP), vitamins C, vitamin E, selenium, N-Acetylcysteine (NAC) estradiol, glutathione, melatonin, resveratrol, flavonoid, carotene, aminoguanidine, or lycopene.

Claim 37: The process of claim 3, wherein said carrier solution comprises photoactive agents selected from the group comprising a xanthene dye, naphthalimide compounds, riboflavin-5-phosphate, N-hydroxypyridine-2-(1H)-thione, N-(20-ethylaminoethyl)-4-amino-1,8-naphthalimide, bis-diazopyruvamide—N,N9-bis(3-diazopyruvoyl)-2,29-(ethylenedioxy)bis-(ethylamine) (DPD); diazopyruvoyl (DAP); methylene blue, erythrosin, phloxime, thionine, methylene green, rose Bengal, acridine orange, xanthine dye, and thioxanthine dyes, ethyl eosin, eosin Y, or a combination comprising at least one of the foregoing photoactive groups.

Claim 38: The process according to 37, wherein said photoactive agent is activated by UV light or a laser.

Claim 39: The process of claim 3, wherein the weight percentage of said carrier in said tissue repair implant is no more than 20% in the dry state.

Claim 40: The process of claim 3, wherein the weight percentage said carrier in said tissue repair implant is no more than 5% in the dry state.

Claim 41: The process of claim 3, wherein said natural or synthetic bone fragments materials comprise non-demineralized bone, partially demineralized bone, demineralized bone, ceramics, hydroxyapatite, calcium phosphate, calcium sulfate, calcium carbonate, or a combination of two or more of the above.

Claim 42: The process of claim 3, said natural bone fragments are from human or animals.

Claim 43: The process of claim 3, wherein said natural or synthetic bone fragments are in the form of particles, fibers, rods, or cubes.

Claim 44: The process of claim 40, wherein said particles have an average particle size of about 125 micron to about 2000 micron.

Claim 45: The process of claim 40, wherein said fibers have an average width of 0.1 to 2 mm and an average length of about 0.3 mm to about 100 mm.

Claim 46: The process of claim 40, wherein said rods have average width of 0.5 to 5 mm and an average length of about 1 mm to about 100 mm.

Claim 47: The process of claim 40, wherein said cubes have an average volume of about 0.001 mm³ to an average volume of about 1000 mm³.

Claim 48: The process of claim 37, wherein said demineralized bone have a residual calcium content from about 1% to about 4%.

Claim 49: The process of claim 3, wherein the weight percentage of said natural or synthetic bone fragments in said tissue repair implant is about 0.1% to about 95% in the dry state.

Claim 50: The process of claim 3, wherein the weight percentage of said natural or synthetic bone fragments in said tissue repair implant is about 10% to about 90% in the dry state.

Claim 51: The process of claim 3, wherein the weight percentage of said natural or synthetic bone fragments in said tissue repair implant is about 30% to about 80% in the dry state.

Claim 52: The process of claim 3, wherein said crudely fragmented connective tissue is from tissues that are cleaned, disinfected, optionally devitalized to remove cellular components, and optionally freeze dried

Claim 53: The process of claim 3, wherein said crudely fragmented connective tissue is from tissues comprising fascia, cartilage, ligament, tendon, pericardium, muscle, urethra, small intestine, dermis, demineralized bone, partial demineralized bone, or non demineralized bone.

Claim 54: The process of claim 3, wherein the average diameter of said crudely fragmented connective tissue is from about 0.01 to about 5 mm.

Claim 55: The process of claim 3, wherein the weight percentage of said crudely fragmented connective tissue in said tissue repair implant is about 0.25% to about 95% in the dry state.

Claim 56: The process of claim 3, wherein the weight percentage of said crudely fragmented connective tissue in said tissue repair implant is about 0.5% to about 90% in the dry state.

Claim 57: The process of claim 3, wherein said tissue repair mixture is placed in a mold before said freezing or freeze-drying step.

Claim 58: The process of claim 3, wherein said tissue repair mixture is optionally processed under negative hydrostatic pressure before being frozen to increase porosity.

Claim 59: The process of claim 3, wherein said treatment solution comprises ionic, enzymatic, or chemical crosslinking agents; or photoactive agents.

Claim 60: The process of claim 59, wherein said ionic crosslinking agents comprise one or more metal ions selected from the group consisting of calcium, barium, aluminum, strontium, copper, zinc, magnesium, manganese, cobalt, or iron.

Claim 61: The process of claim 59, wherein said enzymatic crosslinking agents comprise transglutaminase, ethylenediamine, lysyl oxidase family, hexamethylene diisocyanate (HMDIC), dimethyl suberimidate (DMS), or dimethyl-3-3'-dithiobispropionimide (DTBP).

Claim 62: The process of claim 59, wherein said chemical crosslinking agents comprise glutaraldehyde, glyceraldehyde, genipin, glucose or ribose, poly(ethylene glycol) diepoxide crosslinker, poly(ethylene glycol) diglycidyl ether, EDC and NHS, or acryl azide.

Claim 63: The process of claim 59, wherein said treatment solution comprises natural and or synthetic polymers selected from the group comprising native or modified collagen, gelatin, agarose, modified hyaluronic acid, fibrin, chitin, biotin, avidin, demineralized bone matrix, MATRIGEL[®], HUMAN EXTRACELLULAR MATRIX[®], proteoglycans, laminin, fibronectin, elastin, heparin, glycerol, sucrose octasulfate, polyethylene glycol, polymethylmethacrylate, polyurethane, acryloilmorpholine, N,N-dimethyl acrylamide, N-vinyl pyrrolidone and tetrahydrofurfuryl methacrylate, hydroxyapatite, cross-linkage or functionalization of hyaluronan-based collagen and alginate, polyurethane, or polylactic acid, or a combination comprising at least one of the foregoing polymers.

Claim 64: The process of claim 11, wherein said one or more bioactive supplements are incorporated in the treatment solution.

Claim 65: The process of claim 12, wherein said one or more agents that have bioactive supplement binding site(s) is incorporated in the treatment solution.

Claim 66: The process of claim 3, wherein said treatment solution comprises antioxidants to protect bioactive components from oxygen-radical-induced damage.

Claim 67: The process of claim 3, wherein said treating step comprising one or more treatment solutions are conducted simultaneously or sequentially to stabilize the porous sponge-like structure and further entrap said natural or synthetic bone fragments.

Claim 68: The process of claim 3, wherein said treatment is carried out for a duration of about 5 minutes to about 16 hours.

Claim 69: The process of claim 3, wherein said treatment is carried out for a duration of about 15 minutes to about 120 minutes.

Claim 70: The process of claim 3, wherein said treatment is carried out for a duration of about 30 minutes to about 60 minutes.

Claim 71: The process of claim 9, wherein said sterilization is carried out using gamma irradiation, super critical solution, ethylene oxide, or electronic-beam.

Claim 72: A process for repairing a defect comprising implanting said tissue repair implant having a porous sponge-like structure according to claim 3 or 5 into a defect without rehydration to allow said tissue repair implant to absorb blood or fluid as well as autologous cells in situ.

Claim 73: A process for repairing a defect comprising:
rehydrating said tissue repair implant having a porous sponge-like structure according to claim 3 or 5 with a rehydrating solution;
optionally seeding vital cells on said tissue repair implant to render said tissue repair implant vital;
optionally culture said cell-seeded tissue repair implant before implantation;
implanting said tissue repair implant into said defect.

Claim 74: The process of claim 73, wherein said rehydrating solution comprises blood or bone marrow aspirate, platelet rich plasma, enzymes, bioactive supplements, natural polymers, synthetic polymers, photoactive agents, antioxidants, crosslinking agents, antimicrobial agents, vital cells, one or more agents that have bioactive supplement binding site(s), or a mixture of two or more of the above.

Claim 75: The process of claim 73, wherein said vital cells comprise one or more than one type of cells from autologous or allograft bone marrow aspirate; stromal cells from bone marrow, stromal cells from fat, synovium, periosteum, perichondrium, muscle, dermis, umbilical cord blood, and Warton's jelly; or pericytes.

Claim 76: A process for preparing a biologically functional tissue repair implant having a porous sponge-like structure comprising:

- a. producing a connective tissue homogenate from one or more connective tissues;
- b. mixing said connective tissue homogenate with one or more natural or synthetic bone fragments to produce a mixture;
- c. adding a carrier solution to said mixture to produce a tissue repair mixture;
- d. freezing or freeze-drying said tissue repair mixture to produce a porous sponge-like structure and create a three-dimensional framework to entrap said natural or synthetic bone fragments; and
- e. treating said frozen or freeze-dried porous sponge-like structure with one or more treatment solutions to produce a stabilized porous sponge-like structure.

Claim 77: A method of repairing an injured tissue in a subject comprising applying the implant of claim 1 to the injured tissue to promote repair or regeneration of the tissue.

Claim 78: The process according to claim 3, 5, or 76, wherein the process further comprises seeding cells on the implant.

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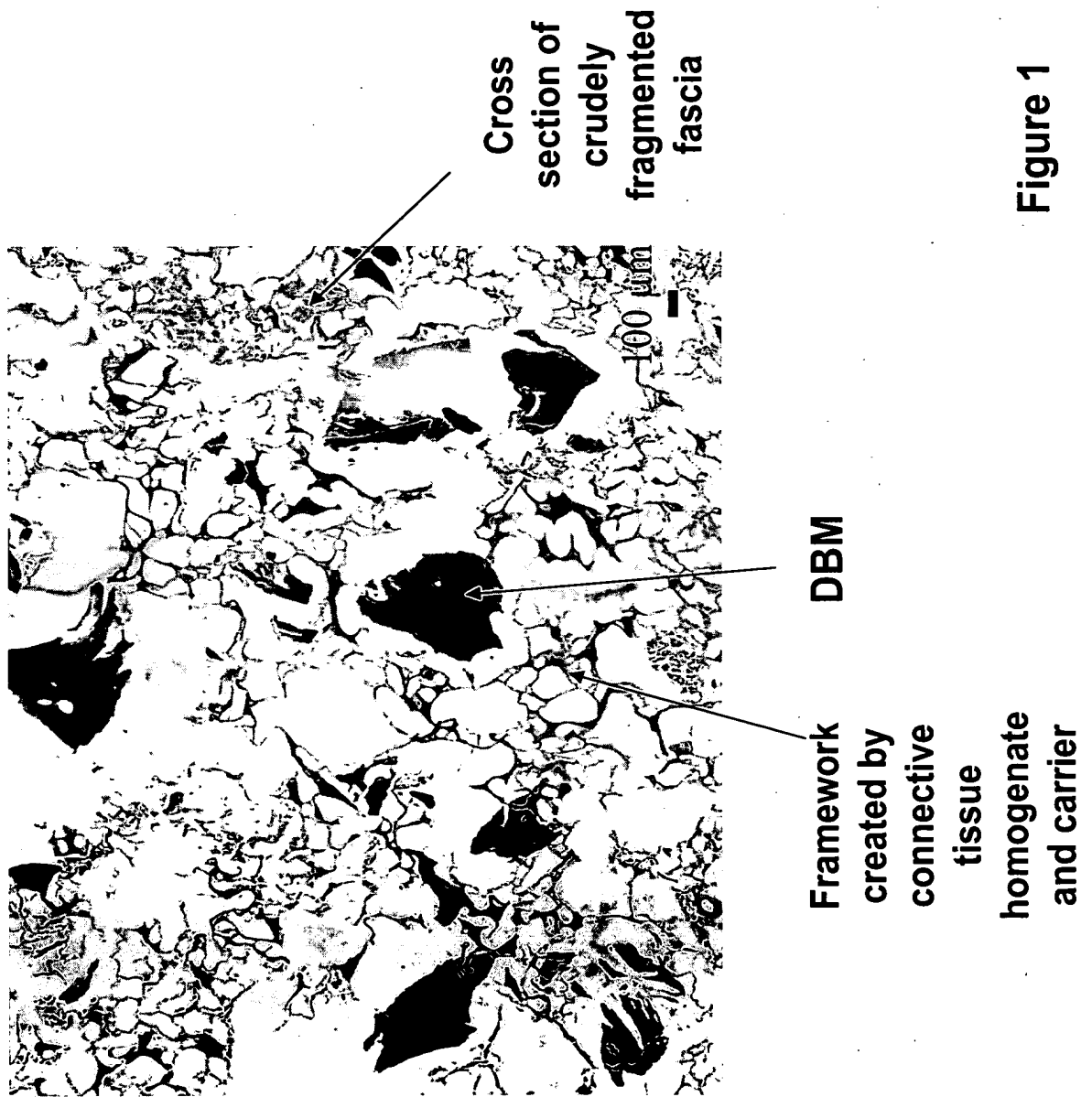


Figure 1

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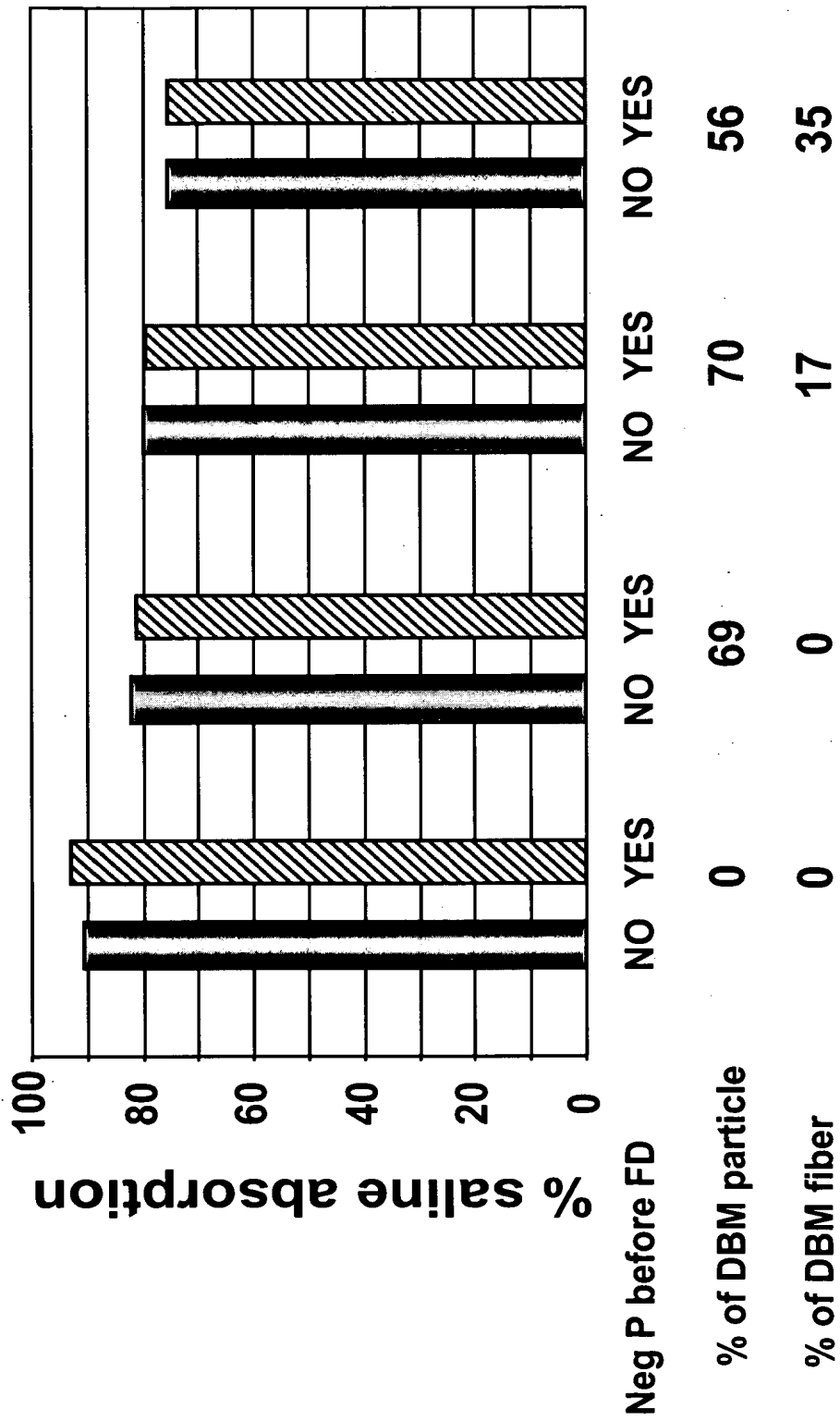


Figure 2

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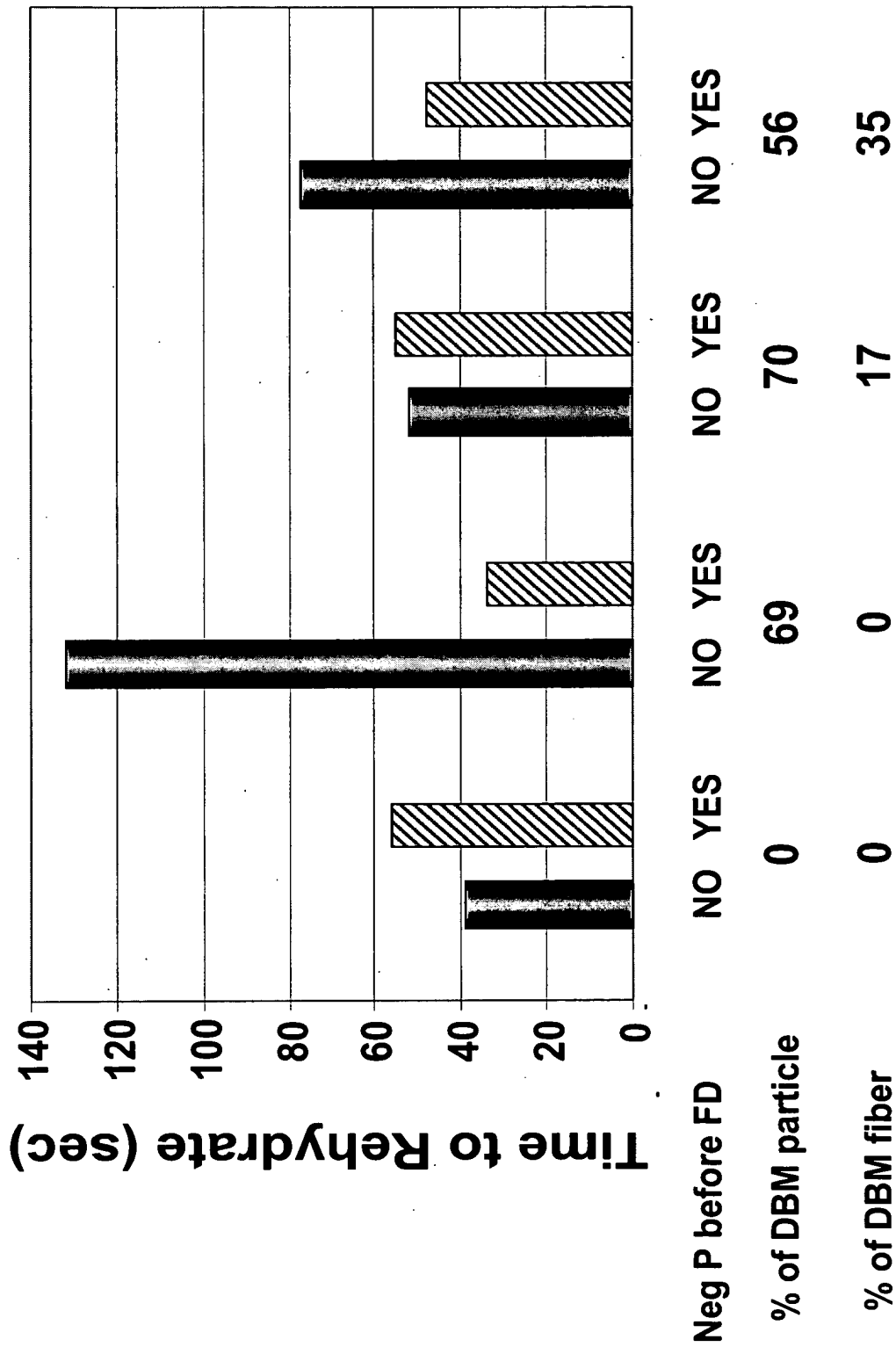


Figure 3

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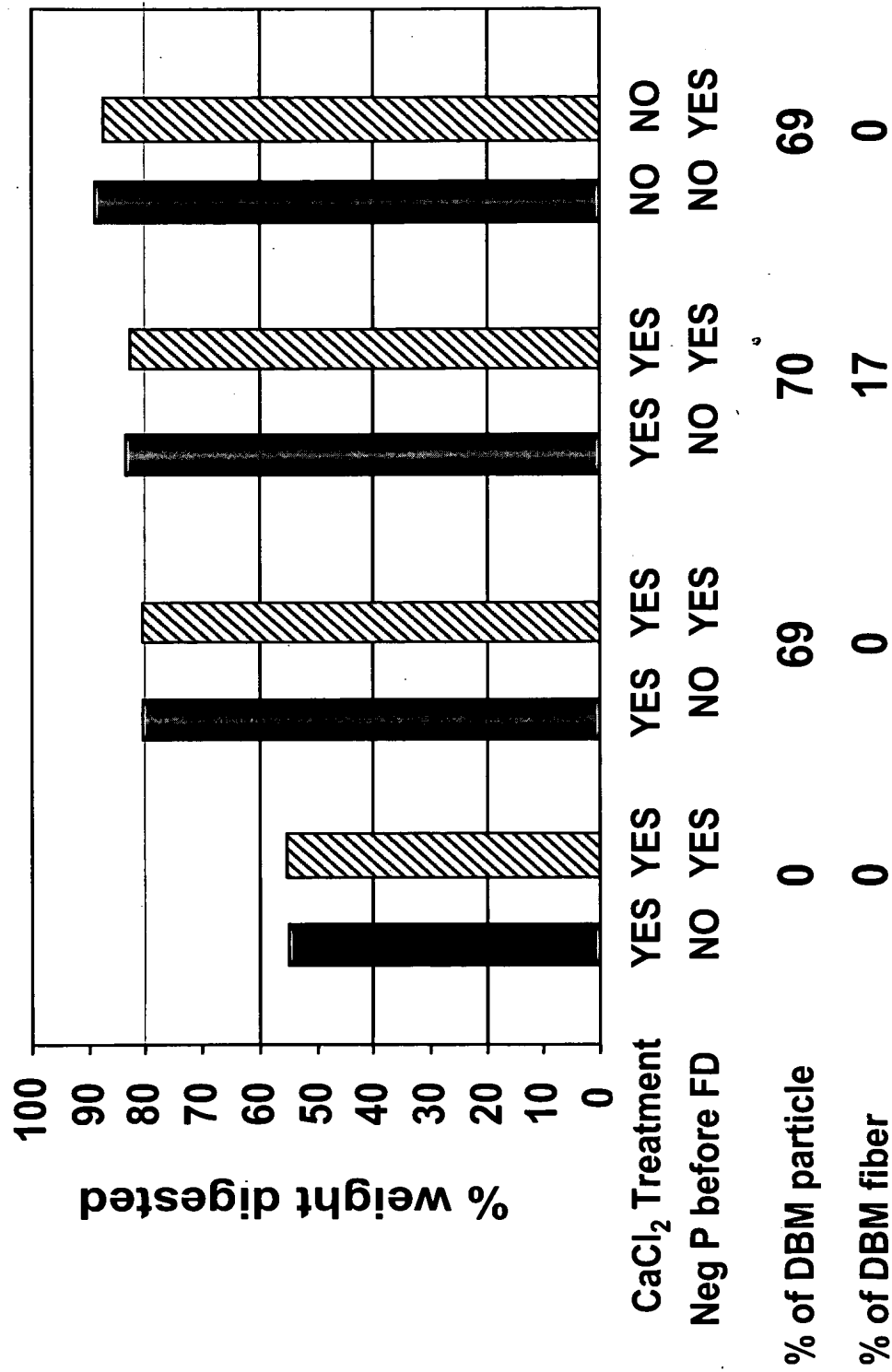


Figure 4

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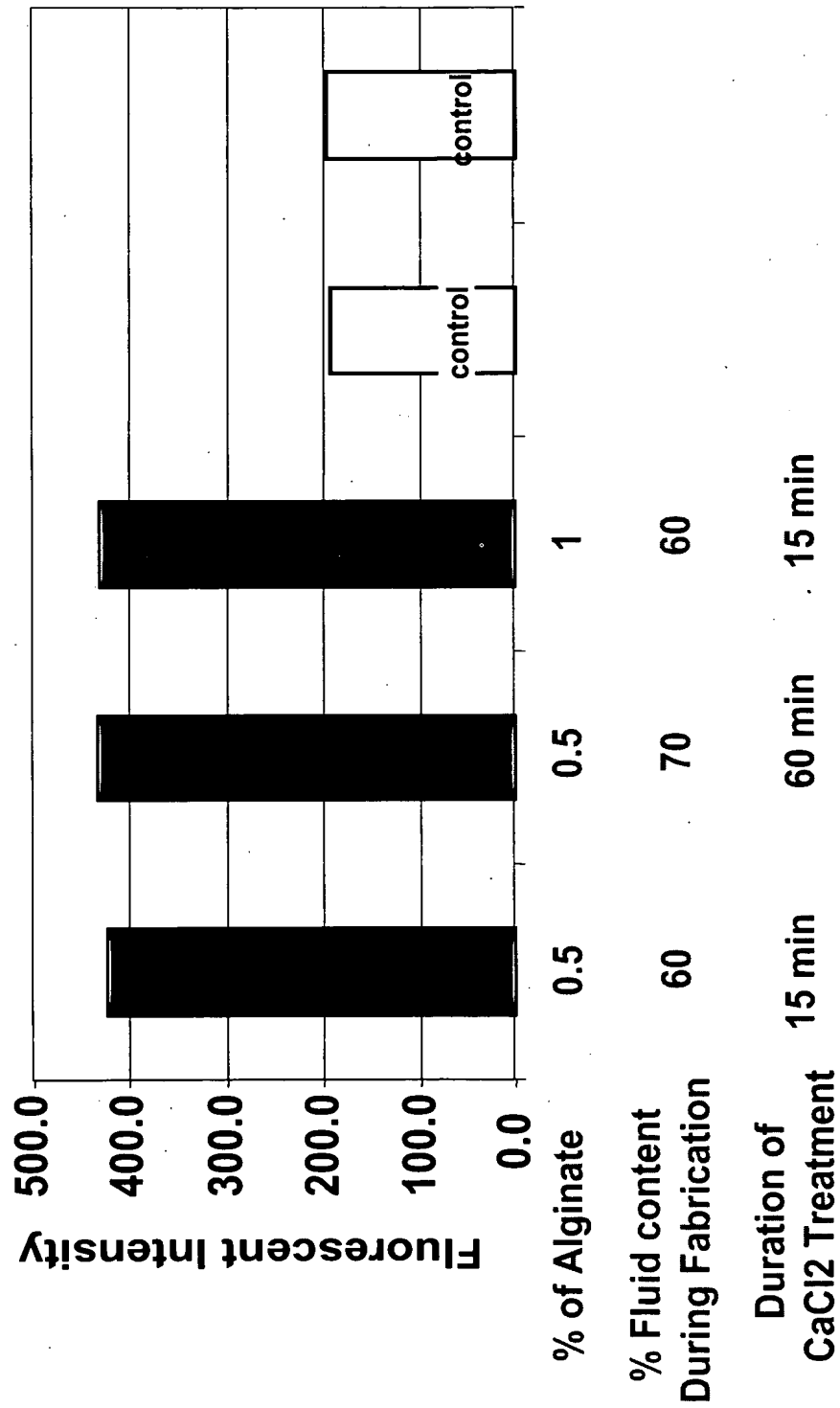


Figure 5

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Figure 6

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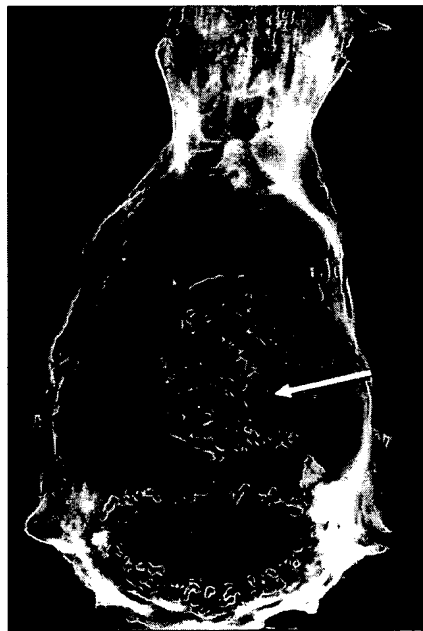


Figure 7

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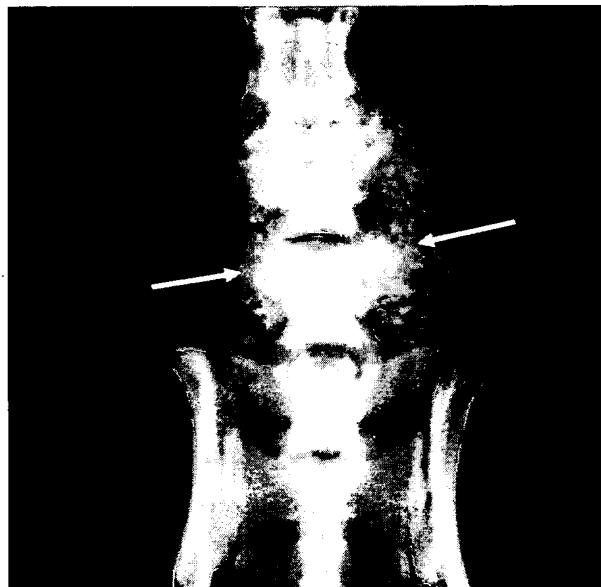


Figure 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2009/004556

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 35/32 (2009.01)

USPC - 424/549

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 35/32 (2009.01)

USPC - 424/549

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MicroPatent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	US 2008/0069893 A1 (MASINAEI et al) 20 March 2008 (20.03.2008) entire document	3-6, 8-14, 19-21, 23-28, 35, 39-46, 49-51, 53-57, 59, 64-65, 67, 71, 76 1-2, 7, 15-18, 22, 29-34, 36-38, 47-48, 52, 58, 60-63, 66, 68-70, 72-75, 77-78
Y	US 2004/0059364 A1 (GASKINS et al) 25 March 2004 (25.03.2004) entire document	1-2, 77
Y	US 5,607,474 A (ATHANASIOU et al) 04 March 1997 (04.03.1997) entire document	15-17, 58
Y	US 2008/0077251 A1 (CHEN et al) 27 March 2008 (27.03.2008) entire document	7, 18, 22, 29-34, 36-38, 47-48, 52, 61-63, 66, 68-70, 72-75, 78
Y	US 2008/0132991 A1 (PINCHUK et al) 05 June 2008 (05.06.2008) entire document	60

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 September 2008

Date of mailing of the international search report

01 OCT 2009

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

Electronic Patent Application Fee Transmittal

Application Number:	14776106			
Filing Date:	14-Sep-2015			
Title of Invention:	ALIGNED FIBER AND METHOD OF USE THEREOF			
First Named Inventor/Applicant Name:	Michael FRANCIS			
Filer:	Glenn E. Murphy/Nicole May			
Attorney Docket Number:	LNH-152US			
Filed as Small Entity				
Filing Fees for U.S. National Stage under 35 USC 371				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
RCE- 1ST REQUEST	2801	1	650	650
Total in USD (\$)				650

Electronic Acknowledgement Receipt

EFS ID:	33301747
Application Number:	14776106
International Application Number:	
Confirmation Number:	1091
Title of Invention:	ALIGNED FIBER AND METHOD OF USE THEREOF
First Named Inventor/Applicant Name:	Michael FRANCIS
Customer Number:	23122
Filer:	Glenn E. Murphy/Nicole May
Filer Authorized By:	Glenn E. Murphy
Attorney Docket Number:	LNH-152US
Receipt Date:	27-JUL-2018
Filing Date:	14-SEP-2015
Time Stamp:	13:04:38
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	\$ 650
RAM confirmation Number	072718INTEFSW13053100
Deposit Account	180350
Authorized User	Nicole May

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Preliminary Amendment	PreliminaryAmendment.pdf	48795	no	3
			f74140c7d2d1914627434144ce5975f91adc1c0		
Warnings:					
Information:					
2	Request for Continued Examination (RCE)	RCE.pdf	137007	no	3
			025ba1763f16f14354e6879c62d7d17323b832cf		
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
3	Drawings-only black and white line drawings	ReplacementDrawing.pdf	229843	no	1
			9262cf974dd214749e86143496139d9a4d620767		
Warnings:					
Information:					
4	Transmittal Letter	IDS.pdf	37206	no	2
			901ae3667bdc9dec435b68411537062255253a6d		
Warnings:					
Information:					
5	Information Disclosure Statement (IDS) Form (SB08)	PTOSB08a.pdf	228497	no	5
			d352db27e4f14dc7f96d1ba924a0b6f06c0fe147		
Warnings:					
Information:					
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6	Foreign Reference	WO2012027592A2.pdf	1300880	no	25
			142220fdf5827e50abf8dfd8cdb69261ed0ff7c		

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Information:					
7	Foreign Reference	WO2013033680A1.pdf	5289669	no	106
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Warnings:					
Information:					
8	Foreign Reference	WO2013119873A1.pdf	3841700	no	63
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Warnings:					
Information:					
9	Foreign Reference	WO2010016942A1.pdf	3275961	no	70
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Information:					
10	Non Patent Literature	Anderson_Biodegradation.pdf	1154129	no	20
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Warnings:					
Information:					
11	Non Patent Literature	Ayres_Measuring.pdf	9908173	no	20
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12	Non Patent Literature	Behravesht_Synthetic.pdf	2631526	no	12
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13	Non Patent Literature	Cleek_Microparticles.pdf	452496	no	10
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14	Non Patent Literature	Lu_Polymeric.pdf	3201013 b93b0fc4ac6a4cead7009a4796da4d73d7da4dc2	no	15
Warnings:					
Information:					
15	Non Patent Literature	Schnaper_Regulation.pdf	1716032 f68112aaa5d8bb9e47649cccb6d031f3c6389173a	no	9
Warnings:					
Information:					
16	Fee Worksheet (SB06)	fee-info.pdf	30255 3ea6e279db60892548ecfe54df6285cfaed94a37	no	2
Warnings:					
Information:					
Total Files Size (in bytes):			33483182		
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

Appln. No.: 14/776,106
Amendment Dated July 27, 2018
Reply to Notice of Allowance of June 12, 2018

LNH-152US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. No: 14/776,106
Applicant: LifeNet Health
Filed: September 14, 2015
Title: ALIGNED FIBER AND METHOD OF USE THEREOF
TC/A.U.: 1653
Examiner: Douglas F. White
Confirmation No.: 1091
Docket No.: LNH-152US

PRELIMINARY AMENDMENT

Mail Stop Issue Fee
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Applicants amend this application as follows:

- ☐ **Amendments to the Specification** begin on page of this paper.
- ☐ **Amendments to the Claims** are reflected in the listing of claims which begins on page of this paper.
- ☒ **Amendments to the Drawings** begin on page **2** of this paper and include an attached replacement sheets.
- ☐ **Amendments to the Abstract** are on page of this paper. A clean version of the Abstract is on page of this paper.
- ☒ **Remarks/Arguments** begin on page **3** of this paper.

Appln. No.: 14/776,106
Amendment Dated July 27, 2018
Reply to Notice of Allowance of June 12, 2018

LNH-152US

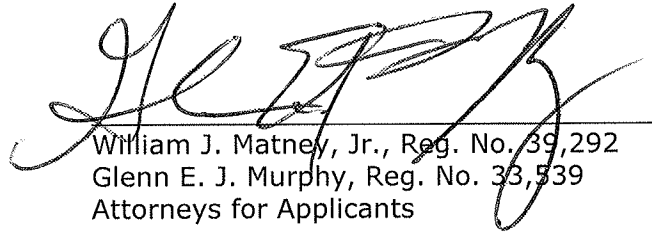
Amendments to the Drawings:

Please cancel original Figure 8 and enter the attached replacement drawing sheet.

Remarks/Arguments:

Figure 8 is hereby amended to correct a labeling error. In that this error is obvious, no new matter is added to this application by its correction here. Examination and early allowance are earnestly solicited.

Respectfully submitted,



William J. Matney, Jr., Reg. No. 39,292
Glenn E. J. Murphy, Reg. No. 33,539
Attorneys for Applicants

WJM/GEJM/nm

Attachment: Figures 8A and 8B (1 sheet)

Dated: July 27, 2018

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Suite 350
King of Prussia, PA 19406
(610) 407-0700

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/776,106	Filing Date 09/14/2015	☐ To be Mailed
---	---	----------------------------------	---------------------------------------

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO**APPLICATION AS FILED – PART I**

(Column 1)

(Column 2)

FOR	NUMBER FILED	NUMBER EXTRA	RATE (\$)	FEE (\$)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X \$ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X \$ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

(Column 1)

(Column 2)

(Column 3)

AMENDMENT	07/27/2018	CLAIMS REMAINING AFTER AMENDMENT	MINUS	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)	ADDITIONAL FEE (\$)
	Total (37 CFR 1.16(i))	* 15	Minus	** 20	= 0	X \$50 =	0
	Independent (37 CFR 1.16(h))	* 1	Minus	*** 3	= 0	X \$230 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
TOTAL ADD'L FEE							0

(Column 1)

(Column 2)

(Column 3)

AMENDMENT	CLAIMS REMAINING AFTER AMENDMENT	MINUS	HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE (\$)	ADDITIONAL FEE (\$)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X \$ =
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X \$ =
	☐ Application Size Fee (37 CFR 1.16(s))					
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))					
TOTAL ADD'L FEE						

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
LINDA HUMES

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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Application Number: 14776106

Document Date: 07/27/2018

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NOTICE OF ALLOWANCE AND FEE(S) DUE

23122 7590 08/15/2018
RATNERPRESTIA
2200 Renaissance Blvd
Suite 350
King of Prussia, PENNSYLVANIA 19406
UNITED STATES OF AMERICA

EXAMINER

WHITE, DOUGLAS F

ART UNIT

PAPER NUMBER

1653

DATE MAILED: 08/15/2018

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/776,106	09/14/2015	Michael FRANCIS	LNH-152US	1091

TITLE OF INVENTION: ALIGNED FIBER AND METHOD OF USE THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	\$500	\$0.00	\$0.00	\$500	11/15/2018

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: **Mail** **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

23122 7590 08/15/2018
RATNERPRESTIA
 2200 Renaissance Blvd
 Suite 350
 King of Prussia, PENNSYLVANIA 19406
 UNITED STATES OF AMERICA

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/776,106	09/14/2015	Michael FRANCIS	LNH-152US	1091

TITLE OF INVENTION: ALIGNED FIBER AND METHOD OF USE THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	\$500	\$0.00	\$0.00	\$500	11/15/2018

EXAMINER	ART UNIT	CLASS-SUBCLASS
WHITE, DOUGLAS F	1653	435-395000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

☐ Issue Fee

☐ Publication Fee (No small entity discount permitted)

☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): (**Please first reapply any previously paid issue fee shown above**)

☐ A check is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29

☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____

Date _____

Typed or printed name _____

Registration No. _____



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/776,106	09/14/2015	Michael FRANCIS	LNH-152US	1091
23122	7590	08/15/2018	EXAMINER	
RATNERPRESTIA 2200 Renaissance Blvd Suite 350 King of Prussia, PENNSYLVANIA 19406 UNITED STATES OF AMERICA			WHITE, DOUGLAS F	
			ART UNIT	PAPER NUMBER
			1653	
DATE MAILED: 08/15/2018				

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 14/776,106	Applicant(s) FRANCIS et al.	
	Examiner DOUGLAS F WHITE	Art Unit 1653	AIA Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to the communication filed on 27 July 2018.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are See Continuation Sheet. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov**.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☐ All b) ☐ Some *c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. _____.

3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file areply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892) 2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____. 3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____. 4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____. 	5. ☒ Examiner's Amendment/Comment 6. ☐ Examiner's Statement of Reasons for Allowance 7. ☐ Other _____.
--	--

/DOUGLAS F WHITE/ Examiner, Art Unit 1653	/CHRISTOPHER R TATE/ Primary Examiner, Art Unit 1655
--	---

Continuation of 3. The allowed claim(s) is/are: 1,5-6,11-12,17,19-22,27-28 and 31-33

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after allowance or after an Office action under *Ex Parte Quayle*, 25 USPQ 74, 453 O.G. 213 (Comm'r Pat. 1935). Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, prosecution in this application has been reopened pursuant to 37 CFR 1.114.

Applicant's submission filed on 27 July 2018 has been entered.

Drawings

The drawings received on 27 July 2018 are acceptable.

Examiner's Comment

The claimed invention has been examined on the merits and found allowable – as amended within the Examiner's Amendment set forth below. Please also refer to the Rejoinder statement below. In addition, the documents cited within Information Disclosure Statement (IDS) filed 27 July 2018 have been considered by the examiner. An examiner-signed copy of the IDS is attached.

Rejoinder

Claims 1, 5-6, 11-12, 17, and 19-22 are directed to an allowable product. Pursuant to the procedures set forth in MPEP § 821.04(B), claims 27-28 and 31-33, directed to the process of making or using an allowable product, previously withdrawn from consideration as a result of a restriction requirement, are hereby rejoined and fully examined for patentability under 37 CFR 1.104.

Because all claims previously withdrawn from consideration under 37 CFR 1.142 have been rejoined, **the restriction requirement as set forth in the Office action mailed on [4] is hereby withdrawn.** In view of the withdrawal of the restriction requirement as to the rejoined inventions, applicant(s) are advised that if any claim presented in a continuation or divisional application is anticipated by, or includes all the limitations of, a claim that is allowable in the present application, such claim may be subject to provisional statutory and/or nonstatutory double patenting rejections over the claims of the instant application. Once the restriction requirement is withdrawn, the provisions of 35 U.S.C. 121 are no longer applicable. See *In re Ziegler*, 443 F.2d 1211, 1215, 170 USPQ 129, 131-32 (CCPA 1971). See also MPEP § 804.01.

Examiner's Amendment

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it **MUST** be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Glenn Murphy on 06 June 2018.

IN THE CLAIMS:

In claim 27, at line 2, the phrase “in condition” has been omitted and replaced with the phrase --under conditions--.

In claim 31, at line 2, the phrase “in conditions” has been omitted and replaced with the phrase --under conditions--.

Conclusion

Claims 1, 5-6, 11-12, 17, 19-22, 27-28 and 31-33 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to DOUGLAS F WHITE whose telephone number is (571)270-5561. The examiner can normally be reached on Monday-Thursday 8am-6:30pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at <http://www.uspto.gov/interviewpractice>.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sharmila G. Landau can be reached on 571-272-0614. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/DOUGLAS F. WHITE/
Examiner, Art Unit 1653

/CHRISTOPHER R TATE/
Primary Examiner, Art Unit 1655

Issue Classification 	Application/Control No. 14/776,106	Applicant(s)/Patent Under Reexamination FRANCIS et al.
	Examiner DOUGLAS F WHITE	Art Unit 1653

CPC						
Symbol					Type	Version
A61L	/	27	/	18	F	2013-01-01
A61L	/	27	/	24	I	2013-01-01
A61L	/	27	/	26	I	2013-01-01
A61L	/	27	/	3604	I	2013-01-01
A61L	/	27	/	3633	I	2013-01-01
A61L	/	27	/	38	I	2013-01-01
A61L	/	27	/	383	I	2013-01-01
A61L	/	27	/	56	I	2013-01-01
D01D	/	5	/	0076	I	2013-01-01
D01D	/	5	/	0092	I	2013-01-01
C12N	/	5	/	0618	I	2013-01-01
A61F	/	2	/	08	A	2013-01-01
A61F	/	2240	/	001	A	2013-01-01
A61L	/	2400	/	12	A	2013-01-01
C12N	/	2533	/	30	A	2013-01-01
C12N	/	2533	/	54	A	2013-01-01
C12N	/	2533	/	90	A	2013-01-01

CPC Combination Sets								
Symbol					Type	Set	Ranking	Version
A61L	/	27	/	26	I	1	1	2013-01-01
C08L	/	67	/	04	I	1	2	2013-01-01
A61L	/	27	/	18	I	2	1	2013-01-01
C08L	/	67	/	04	I	2	2	2013-01-01

/DOUGLAS F WHITE/ Examiner, Art Unit 1653 (Assistant Examiner)		01 August 2018 (Date)	Total Claims Allowed: 15	
/CHRISTOPHER R TATE/ Primary Examiner, Art Unit 1655 (Primary Examiner)		01 August 2018 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure 5

Issue Classification 	Application/Control No. 14/776,106	Applicant(s)/Patent Under Reexamination FRANCIS et al.	
	Examiner DOUGLAS F WHITE	Art Unit 1653	

INTERNATIONAL CLASSIFICATION					
CLAIMED					
A61K	/	38	/	17	
C07K	/	1	/	00	
C07K	/	14	/	00	
C07K	/	16	/	00	
C07K	/	17	/	00	
C09H	/	1	/	00	
NON-CLAIMED					
	/		/		

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS
435	395

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

/DOUGLAS F WHITE/ Examiner, Art Unit 1653 (Assistant Examiner)	01 August 2018 (Date)	Total Claims Allowed: 15	
/CHRISTOPHER R TATE/ Primary Examiner, Art Unit 1655 (Primary Examiner)	01 August 2018 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure 5

<i>Issue Classification</i> 	Application/Control No. 14/776,106	Applicant(s)/Patent Under Reexamination FRANCIS et al.
	Examiner DOUGLAS F WHITE	Art Unit 1653

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47															
CLAIMS															
Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

/DOUGLAS F WHITE/ Examiner, Art Unit 1653 (Assistant Examiner)	01 August 2018 (Date)	Total Claims Allowed: 15	
/CHRISTOPHER R TATE/ Primary Examiner, Art Unit 1655 (Primary Examiner)	01 August 2018 (Date)	O.G. Print Claim(s) 1	O.G. Print Figure 5

Search Notes 	Application/Control No. 14/776,106	Applicant(s)/Patent Under Reexamination FRANCIS et al.
	Examiner DOUGLAS F WHITE	Art Unit 1653

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST- Inventor and text search	12/7/2017	DFW
PALM- Inventor search	12/7/2017	DFW
Updated	06/06/2018	DFW
Updated	08/01/2018	DFW

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
EAST search	attached	06/06/2018	DFW
EAST search	attached	08/01/2018	DFW

	/DOUGLAS F WHITE/ Examiner.Art Unit 1653
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	4	(WO-2010016942-\$ WO-2012027592-\$ WO-2013033680-\$ WO-2013119873-\$ PP-481997-\$).DID.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2018/08/01 09:56
L2	4	(WO-2010016942-\$ WO-2012027592-\$ WO-2013033680-\$ WO-2013119873-\$).DID.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2018/08/01 09:56
S2	19	((Roy) near2 (Ogle)).INV.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/11/21 16:27
S3	3	"20080213389"	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/11/21 16:29
S4	6	(US-20100233115-\$ US-20110236974-\$ US-20120221025-\$).DID.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/11/21 16:46
S5	185	(electrospun adj fibers) same collagen	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/11/21 16:48
S6	16634	"I6" and calcium	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/11/21 16:49
S7	18924	"I5" and calcium	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/11/21 16:49
S8	84	S5 and calcium	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/11/21 16:49
S9	36	S8 and ECM	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/11/21 16:50
S10	58	S8 and (ECM or (extracellular adj matrix))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/11/21 16:52
S11	1	((Micheal) near2 (Francis)).INV.	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/07 15:32

S12	186	(electrospun adj fibers) same collagen	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/07 15:41
S13	84	S12 and calcium	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/07 15:41
S14	58	S13 and (ECM or (extracellular adj matrix))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/07 15:41
S15	42	S14 and native	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/07 15:41
S16	186	(electrospun adj fibers) same collagen	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/10 07:03
S17	84	S16 and calcium	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/10 07:03
S18	58	S17 and (ECM or (extracellular adj matrix))	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/10 07:03
S19	42	S18 and native	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/10 07:03
S20	13	S19 and helical	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/10 07:03
S21	6	S20 and Fourier	US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT	OR	OFF	2017/12/10 07:05

EAST Search History (I nterference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S22	2	(electrospun adj fibers) same collagen.clm.	USPAT	OR	OFF	2018/06/07 12:08
S23	74	(electrospun adj fibers) and collagen.clm.	USPAT	OR	OFF	2018/06/07 12:09
S24	1	S23 and Fourier.clm.	USPAT	OR	OFF	2018/06/07 12:09

8/ 1/ 2018 9:59:20 AM
C:\ Users\ dwhite3\ Documents\ EAST\ Workspaces\ 14776106.wsp

Doc code: IDS

Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (03-15)

Approved for use through 07/31/2016. OMB 0651-0031

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)	Application Number		14776106	
	Filing Date		2015-09-14	
	First Named Inventor	Michael Francis et al.		
	Art Unit	1653		
	Examiner Name	Douglas F. White		
	Attorney Docket Number	LNH-152US		

U.S.PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code ¹	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear
	1					

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U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code ¹	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear
	1					

If you wish to add additional U.S. Published Application citation information please click the Add button.

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Foreign Document Number ³	Country Code ²	Kind Code ⁴	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T ⁵
	1	2012027592	WO	A2	2012-03-01	LifeNet Health		☐
	2	2013033680	WO	A1	2013-03-07	LifeNet Health		☐
	3	2013119873	WO	A1	2013-08-15	LifeNet Health		☐

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
(Not for submission under 37 CFR 1.99)

Application Number		14776106
Filing Date		2015-09-14
First Named Inventor	Michael Francis et al.	
Art Unit	1653	
Examiner Name	Douglas F. White	
Attorney Docket Number	LNH-152US	

4	2010016942	WO	A1	2010-02-11	Lifenet Health	☐
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If you wish to add additional Foreign Patent Document citation information please click the Add button

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T ⁵
	1	Anderson et al., "Biodegradation and Biocompatibility of PLA and PLGA Microspheres", Advanced Drug Delivery Reviews, 1997, Vol. 28, pp. 5-24	☐
	2	Ayres et al., "Measuring Fiber Alignment in Electrospun Scaffolds: A User's Guide to the 2D Fast Fourier Transform Approach," Journal of Biomaterials Science, Polymer Edition, (2008), Vol. 19, No. 5, pp. 603-621	☐
	3	Behraves et al., "Synthetic Biodegradable Polymers for Orthopaedic Applications," Clinical Orthopaedics and Related Research, No. 367S, pp. S118-S125	☐
	4	Cleek et al., "Microparticles of Poly(DL-lactic-co-glycolic acid)/poly(ethylene glycol) Blends for Controlled Drug Delivery," Journal of Controlled Release, Vol. 48 1997 pp. 259-268	☐
	5	Lu et al., "Polymeric Delivery Vehicles for Bone Growth Factors," 2000, Park & Mersny; Controlled Drug Delivery, ACS Symposium Series; American Chemical Society, pp. 124-138	☐
	6	Schnaper et al., "Regulation of Cell Function by Extracellular Matrix," Pediatr. Nephrol, 1993, Vol. 7 - pp. 96-104	☐

If you wish to add additional non-patent literature document citation information please click the Add button

EXAMINER SIGNATURE

Examiner Signature	/DOUGLAS F WHITE/	Date Considered	08/01/2018
--------------------	-------------------	-----------------	------------

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
(Not for submission under 37 CFR 1.99)

Application Number	14776106
Filing Date	2015-09-14
First Named Inventor	Michael Francis et al.
Art Unit	1653
Examiner Name	Douglas F. White
Attorney Docket Number	LNH-152US

¹ See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. ² Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). ³ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁴ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁵ Applicant is to place a check mark here if English language translation is attached.

**INFORMATION DISCLOSURE
STATEMENT BY APPLICANT**
(Not for submission under 37 CFR 1.99)

Application Number	14776106
Filing Date	2015-09-14
First Named Inventor	Michael Francis et al.
Art Unit	1653
Examiner Name	Douglas F. White
Attorney Docket Number	LNH-152US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

- ☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

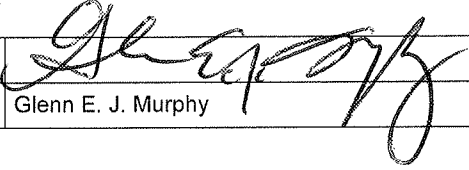
OR

- ☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

- ☐ See attached certification statement.
- ☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.
- ☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature		Date (YYYY-MM-DD)	2018-07-27
Name/Print	Glenn E. J. Murphy	Registration Number	33,539

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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COMMISSIONER FOR PATENTS
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Suite 350
King of Prussia, PA 19406



**Courtesy Reminder for
Application Serial No: 14/776,106**

Attorney Docket No: LNH-152US

Customer Number: 23122

Date of Electronic Notification: 08/21/2018

This is a courtesy reminder that new correspondence is available for this application. If you have not done so already, please review the correspondence. The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

An email notification regarding the correspondence was sent to the following email address(es) associated with your customer number:

PCorrespondence@ratnerprestia.com

To view your correspondence online or update your email addresses, please visit us anytime at <https://portal.uspto.gov/secure/myportal/privatepair>. If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov or call 1-866-217-9197.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: **Mail** Mail Stop ISSUE FEE
 Commissioner for Patents
 P.O. Box 1450
 Alexandria, Virginia 22313-1450
 or **Fax** (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

23122 7890 08/15/2018
 RATNERPRESTIA
 2200 Renaissance Blvd
 Suite 350
 King of Prussia, PENNSYLVANIA 19406
 UNITED STATES OF AMERICA

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILED DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/776,106	09/14/2015	Michael FRANCIS	LNH-152US	1091

TITLE OF INVENTION: ALIGNED FIBER AND METHOD OF USE THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	SMALL	\$500	\$0.00	\$0.00	\$500	11/15/2018

EXAMINER	ART UNIT	CLASS-SUBCLASS
WHITE, DOUGLAS F	1653	435-395000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

2. For printing on the patent front page, list

1. RatnerPrestia

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

2. _____

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

3. _____

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. Use of a Customer Number is required.

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

LifeNet Health

Virginia Beach, VA

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

4b. Payment of Fee(s): (Please first reapply any previously paid issue fee shown above)

☒ Issue Fee

☐ A check is enclosed.

☐ Publication Fee (No small entity discount permitted)

☒ Payment by credit card. Form PTO-2038 is attached.

☐ Advance Order - # of Copies _____

☒ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number 18-0350 (enclose an extra copy of this form).

5. Change in Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

☐ Applicant asserting small entity status. See 37 CFR 1.27

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

☐ Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Glenn E. J. Murphy/

Date October 12, 2018

Typed or printed name Glenn E. J. Murphy

Registration No. 33,539

Electronic Patent Application Fee Transmittal				
Application Number:		14776106		
Filing Date:		14-Sep-2015		
Title of Invention:		ALIGNED FIBER AND METHOD OF USE THEREOF		
First Named Inventor/Applicant Name:		Michael FRANCIS		
Filer:		Glenn E. Murphy/Nicole May		
Attorney Docket Number:		LNH-152US		
Filed as Small Entity				
Filing Fees for U.S. National Stage under 35 USC 371				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	2501	1	500	500

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				500

Electronic Acknowledgement Receipt

EFS ID:	33990189
Application Number:	14776106
International Application Number:	
Confirmation Number:	1091
Title of Invention:	ALIGNED FIBER AND METHOD OF USE THEREOF
First Named Inventor/Applicant Name:	Michael FRANCIS
Customer Number:	23122
Filer:	Glenn E. Murphy/Nicole May
Filer Authorized By:	Glenn E. Murphy
Attorney Docket Number:	LNH-152US
Receipt Date:	12-OCT-2018
Filing Date:	14-SEP-2015
Time Stamp:	09:37:47
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	\$ 500
RAM confirmation Number	101218INTEFSW09382900
Deposit Account	180350
Authorized User	Nicole May

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:
37 CFR 1.20 (Post Issuance fees)

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	IssueFeeTransmittal.pdf	425972	no	1
			098cb8f84ea6a4c884a00f41bd11d2616f041391		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30060	no	2
			8b228e663326bc63c2f2f087e7732aeb08c94c76		
Warnings:					
Information:					
Total Files Size (in bytes):			456032		
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					



APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/776,106	11/27/2018	10137223	LNH-152US	1091

23122 7590 11/07/2018
RATNERPRESTIA
2200 Renaissance Blvd
Suite 350
King of Prussia, PA 19406

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 214 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Michael FRANCIS, Virginia Beach, VA;
LIFENET HEALTH, Virginia Beach, VA
Roy OGLE, Virginia Beach, VA;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

"FEE ADDRESS" INDICATION FORM

Address to:
Mail Stop M Correspondence
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Fax to:
571-273-6500

- OR -

INSTRUCTIONS: The issue fee must have been paid for application(s) listed on this form. In addition, only an address represented by a Customer Number can be established as the fee address for maintenance fee purposes (hereafter, fee address). A fee address should be established when correspondence related to maintenance fees should be mailed to a different address than the correspondence address for the application. **When to check the first box below:** If you have a Customer Number to represent the fee address. **When to check the second box below:** If you have no Customer Number representing the desired fee address, in which case a completed Request for Customer Number (PTO/SB/125) must be attached to this form. For more information on Customer Numbers, see the Manual of Patent Examining Procedure (MPEP) § 403.

For the following listed application(s), please recognize as the "Fee Address" under the provisions of 37 CFR 1.363 the address associated with:



Customer Number:

000197

OR



The attached Request for Customer Number (PTO/SB/125) form.

PATENT NUMBER (if known)	APPLICATION NUMBER
	14/776,106

Completed by (check one):



Applicant/Inventor

/Glenn E. J. Murphy/

Signature



Attorney or Agent of record

33539

(Reg. No.)

Glenn E. J. Murphy

Typed or printed name



Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) (Form PTO/SB/96) is
enclosed or was filed on _____

(Date)

610-407-0700

Requester's telephone number

November 16, 2018

Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. If the assignee is a juristic entity, this form must be signed by a patent practitioner (attorney or agent) of record. Submit multiple forms if more than one signature is required, see below*.



* Total of 1

forms are submitted.

This collection of information is required by 37 CFR 1.363. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 5 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop M Correspondence, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

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1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
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EFS ID:	34329207
Application Number:	14776106
International Application Number:	
Confirmation Number:	1091
Title of Invention:	ALIGNED FIBER AND METHOD OF USE THEREOF
First Named Inventor/Applicant Name:	Michael FRANCIS
Customer Number:	23122
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	SB0047.pdf	1116878	no	2
			3ab71d1a324e6a5b57f428a7f88c9cf3b9b9cc9c		

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