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[Continued on next page]

(54) Title: BIOENGINEERED TISSUE CONSTRUCTS AND METHODS FOR PRODUCING AND USING THEREOF

Figure 14 (cont.)

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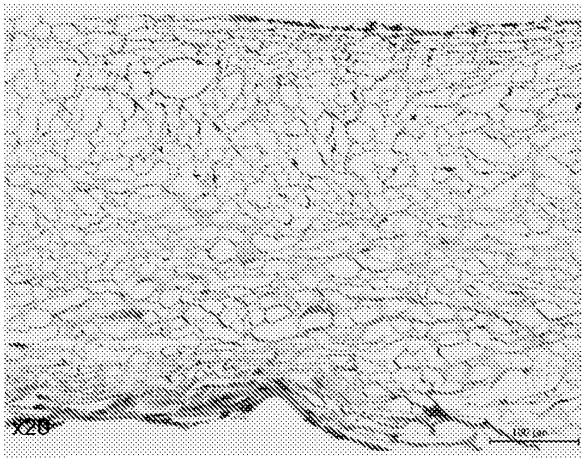

Average Pore Diameter: ~20 µm

(57) Abstract: Bioengineered constructs are formed from cultured cells induced to synthesize and secrete endogenously produced extracellular matrix components without the requirement of exogenous matrix components or network support or scaffold members. The bioengineered constructs of the invention can be produced with multiple cell types that can all contribute to producing the extracellular matrix. Additionally or alternatively, one of the multiple cell types can be delivered to a site in the body via the endogenously produced extracellular matrix components to achieve various therapeutic benefits.

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BIOENGINEERED TISSUE CONSTRUCTS AND METHODS FOR PRODUCING AND USING THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Application No. 61/347,725, filed on May 24, 2010, U.S. Provisional Application No. 61/337,938, filed on February 12, 2010, and U.S. Provisional Application No. 61/295,073, filed on January 14, 2010; the entire contents of each of which are expressly incorporated herein by reference.

BACKGROUND OF THE INVENTION

Bone, cartilage, tendon, ligament, muscle, adipose, and marrow stroma are examples of mesenchymal tissues (*i.e.*, tissues that differentiate from mesenchymal stem cells).

Mesenchymal tissues may be injured during surgery or they may develop disease from a genetic disorder or environmental perturbation.

Accordingly, new therapies for repairing diseased or damaged tissues are needed.

SUMMARY OF THE INVENTION

Featured herein are bioengineered constructs comprising extracellular matrix (ECM) in forms., which are optimized for particular therapeutic uses. Certain constructs are comprised of extracellular matrix produced by cultured mesenchymal stem cells (MSCs). Certain constructs also comprise the cells that produce the matrix. In certain constructs, the cells have been devitalized. In other constructs, the cells, which produce the extracellular matrix have been removed to produce decellularized constructs.

Certain constructs have a thickness of at least about 30 μm. Certain constructs include pores having an average diameter in the range of 10 to 100 um. Certain constructs have an average Fmax of at least 0.4 Newtons. Certain constructs have an ultimate tensile strength (UTS) of at least 0.4 Megapascals. Certain constructs have a plastic deformation tolerance of at least 0.4 times the initial length.

The ECM in the constructs may be further processed (*e.g.*, dehydrated, crosslinked, contracted, micronized, sterilized, etc.) or further combined with other biologically active substances or support materials (*e.g.*, silk, an adhesive, etc.) for preparation of therapeutic products.

Further featured are methods for making and modifying the bioengineered

constructs, including methods to control construct thickness, pore size, and composition.

The bioengineered constructs described herein can be administered to subjects to enhance the vitality, growth and/or repair of soft tissue, including for treatment of chronic or acute wounds.

Other features and advantages will become apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1B show a time course analysis of extracellular matrix formation rate by MSCs between days 5 and 12 (Figure 1A) or between days 12 and 18 (Figure 1B). n=9 (3 independent constructs per group with 3 measurements per construct). A trend line and slope equation are shown.

Figure 2 shows a correlation between increasing bioengineered construct thickness as a function of increased TGF-alpha concentration. No TGF-alpha: 0 ng/mL; 1.5x: 30 ng/mL TGF-alpha; 5x: 100 ng/mL TGF-alpha; and 10x: 200 ng/mL TGF-alpha. n=9 (3 independent constructs per group with 3 measurements per construct), except for 1.5x and 10x where n=6 (2 independent constructs per group and 3 measurements per construct).

Figure 3 shows a correlation between decreasing bioengineered construct thickness as a function of increased Prostaglandin 2 (PGE_2) concentration having a constant amount of 20 ng/mL TGF-alpha. No PGE_2: 0 ng/mL; 5x: 19 ng/mL PGE_2; 10x: 38 ng/mL PGE_2; and 50x: 190 ng/mL PGE_2. n=9 (3 independent constructs per group with 3 measurements per construct).

Figure 4 shows a correlation between increasing bioengineered construct thickness as a function of increased TGF-alpha concentration and cell seeding density across bioengineered constructs derived from MSCs of different cell types (HDF: neonatal human dermal fibroblasts; HUCPVC: human umbilical cord perivascular cells; BM-MSC: bone marrow derived mesenchymal stem cells; and Pre-Adipo: pre-adipocytes). Chemically defined cell culture media described in Example 1 was used (*e.g.*, 200 ng/mL TGF-alpha) and seeding densities were 30 x 10^6 cells per 75 mm insert, which is equivalent to 9.6 x 10^6 cells per 24 mm insert. Matrix thickness measurements collected from hematoxylin and eosin stained sections fixed after 18 days in culture. Bars (mean ± S.D, n=12) represent the average thickness of n=3 independent constructs imaged in 4 separate locations.

Figures 5A-5B show representative hematoxylin and eosin stained, Masson's Trichome/Goldner (MTG) stained, and SEM sections of bioengineered constructs derived

from MSCs of different cell types (HDF: neonatal human dermal fibroblasts; HUCPVC: human umbilical cord perivascular cells; BM-MSC: bone marrow derived mesenchymal stem cells; and Pre-Adipo: pre-adipocytes) after 18 days in culture. Chemically defined cell culture media described in Example 1 was used (*e.g.*, 200 ng/mL TGF-alpha) and seeding densities were 30 x 10^6 cells per 75 mm insert, which is equivalent to 9.6 x 10^6 cells per 24 mm insert. Images captured at 20x magnification.

Figures 6A-6C show representative Fmax, ultimate tensile strength (UTS), and modulus of elasticity properties of bioengineered constructs derived from MSCs of different cell types (HDF-02: neonatal human dermal fibroblasts; HUC-02: human umbilical cord perivascular cells; MSC-02: bone marrow derived mesenchymal stem cells; and PAD-02: pre-adipocytes) after 18 days in culture. Chemically defined cell culture media described in Example 1 was used (*e.g.*, 200 ng/mL TGF-alpha) and seeding densities were 30 x 10^6 cells per 75 mm insert, which is equivalent to 9.6 x 10^6 cells per 24 mm insert. Bars (mean ± S.D, n=9) represent the average Fmax, UTS, modulus of elasticity of 3 independent constructs each tested 3 times.

Figures 7A-7B show a summary of differences in extracellular matrix and adhesion components (Figure 7A; 17 upregulated genes > 2-fold in HUCPVC-derived relative to HDF-derived bioengineered constructs) and growth factors (Figure 7B; 8 upregulated genes > 2-fold in HUCPVC-derived relative to HDF-derived bioengineered constructs) between HUCPVC-derived and HDF-derived bioengineered constructs.

Figures 8A-8D show results of a time-course comparison of IL-6, IL-8, and VEGF levels within the conditioned media generated by various MSC-derived and HDF-derived bioengineered constructs resulting from CBA analyses. The mean and standard deviations are calculated from an average of n=3 conditioned media samples. Quantification of HA levels resulting from ELISA analyses is also shown.

Figure 9 shows results of a cellular migration assay. An indirect 2-D Migration assay comparing closure index as a function of conditioned media collected from various embodiments. Assay is performed on keratinocytes cultured in conditioned media collected from HDF-02 and HUCPVC VCT-02 units at Day 5 and Day 18. The figure consists of representative bright field images of the keratinocytes stained with Acid Fuschin dye after 24 hours of induction in the conditioned media as well as a graphical representation of the closure index values indicating the maximum closure in the HUCPVC VCT-02 Day 5 conditioned media samples.

Figures 10A-10C show results of multilineage potential assays conducted on MSC-derived (HUC-02) and HDF-derived (HDF-02) bioengineered constructs and cells isolated therefrom. Figure 10A shows gene expression data from cells within bioengineered constructs induced using osteogenic induction media using a panel of osteogenic genes. Figure 10B shows gene expression data from cells isolated from bioengineered constructs induced using osteogenic induction media using a panel of osteogenic genes. Figure 10C shows Oil Red O staining results from cells within bioengineered constructs induced using adipogenic induction media.

Figures 11A-11E show representative histological sections and quantitation of alpha-smooth muscle actin (αSMA) staining from a 100% MSC-derived bioengineered constructs (Figure 11A), 50% HUCPVC-50% HDF-derived bioengineered constructs (Figure 11B), 10% HUCPVC-90% HDF-derived bioengineered constructs (Figure 11C), and 100% HDF-derived bioengineered constructs (Figure 11D) after 1 week of subcutaneous implantation into nude mice. Dark areas denote positive staining for αSMA. Figure 11E shows blood vessel quantification within implant area as determined by αSMA positive staining. A total of two animals per group (n = 2) were used for the analysis. The number of αSMA positive vessels was manually counted using 40x objective on a microscope. The number of positive vessels was then normalized to the implant area.

Figure 12 shows independent histological images of bioengineered constructs that have been formalin-fixed immediately after culture.

Figure 13 shows independent histological images of bioengineered constructs that have been allowed to undergo controlled contraction prior to formalin-fixation.

Figures 14A-14G show results of controlling pore sizes within extracellular matrices of bioengineered constructs. Figure 14A shows the different uses of bioengineered constructs according to different average pore diameter properties. Figure 14B shows quantitative analysis of average pore diameters and standard deviations from bioengineered constructs controlled contracted, lyophilized at a final freezing temperatures of -40°C at a rate of 0.1°C per minute, and either not crosslinked, crosslinked with EDC, or crosslinked using DHT methods. Figure 14C shows a representative histological section quantified in Figure 14C. Figure 14D shows a representative histological section of a bioengineered construct ramped to a final freezing temperatures of -10°C at a rate of 0.5°C per minute. Figure 14E shows representative histological sections of bioengineered constructs control contracted and subsequently either air dried (top panel) or lyophilized at

a final freezing temperature of -40°C (bottom panel). Figure 14F shows MSC-derived bioengineered constructs naturally having pores, whereas Figure 14G shows that such average pore diameter can be increased by lyophilizing.

Figures 15A-15E shows the effects on biophysical properties of bioengineered constructs resulting from supplementing chemically defined cultured media with bFGF. Figure 15A shows that bFGF supplementation reduces bioengineered construct thickness. Figure 15B shows the results of bFGF dose response analysis in which sub types of collagen accumulation decreased as bFGF supplementation increased. Figure 15C shows relative levels of both acid- and pepsin-soluble collagen (black) relative to total collagen and other collagen (grey). Sulfated glycosaminoglycan (sGAG; Figure 15D) and hyaluronic acid (HA; Figure 15E) accumulated to lower levels in bFGF-supplemented bioengineered constructs relative to controls.

Figure 16 shows human dermal fibroblasts that have migrated through porous silk scaffolds and are uniformly disposed throughout the silk scaffold.

Figures 17A-17D show stained human umbilical vein endothelial cells atop porous silk scaffolds with devitalized human dermal fibroblasts and its corresponding extracellular matrix, *in vitro*. An *in vitro* angiogenesis assay was developed by examining alignment of stained HUVECs on silk scaffold embodiments. HUVECs were cultured on the silk scaffolds for 11 days and fluorescence images were captured. HUVEC alignment is not visible on the silk scaffold (Figure 17A) or the silk scaffold pre-conditioned in matrix media (Figure 17B), but it is prominent in the silk scaffold with living human dermal fibroblasts (HDF) (Figure 17C) and the silk scaffold with devitalized HDFs (Figure 17D).

DETAILED DESCRIPTION OF THE INVENTION

Featured herein are bioengineered constructs, comprising extracellular matrices (ECM) having defined thickness, pore size, and composition. ECM is known to be secreted by certain cells and is comprised mainly of fibrous proteins, polysaccharides, and other minor constituents. Its components include structural elements such as collagen and elastin, adhesive proteins such as the glycoproteins fibronectin, laminin, vitronectin, thrombospondin I and tenascins, as well as proteoglycans such as decorin, biglycan, chondroitin sulfate and heparin sulfate and glycosaminoglycans (GAG) such as hyaluronic acid (HA).

Different ECMs can be produced by different cells. As compared to fibroblast cells. for example, MSCs have been found to produce a porous ECM. In addition, certain

proteins associated with vascularization (*e.g.*, VEGFα, VEGFC, PDGFβ, PECAM1, CDH5, ANGPT1, MMP2, TIMP1, TIMP3), as well as certain growth factor and adhesion protein, such as hyaluronan, heparin, IL-6, IL-8, vitronectin (VTN), colony-stimulating factor 3 (CSF-3), NCAM1, and CXCL1, appear to be produced in greater amounts in ECM produced by MSCs than by fibroblasts (see *e.g.*, Figure 7)..

The predominant major extracellular matrix component produced by fibroblasts is fibrillar collagen, particularly collagen type I. However, cells also produce other fibrillar and non-fibrillar collagens, including collagen types II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV, XV, XVI, XVII, XVIII, XIX, and others.

The hierarchical network of these ECM components provides a natural environment in which cells can survive and function properly. Cell culture conditions and post-cultured methods, as described herein, can be applied to cell types that are capable of synthesizing and secreting extracellular matrix to produce bioengineered constructs having defined biophysical properties.

I. Controlling bioengineered construct thickness

The thickness of ECM can be optimized for a particular use in vivo. For example, thicker bioengineered constructs may be useful for sites in the body that experience physical agitation (e.g. knees) or for any application for which it is desired that the construct persist *in vivo* for an extended period of time..

The bulk thickness of the ECM confers cohesive tissue-like properties that are resistant to physical damage, such as tearing or cracking.. Appropriate ECMs should have a thickness, which is at least about 30 μm, 40 μm, 50 μm, 60 μm, 70 μm, 80 μm, 90 μm, 100 μm, 110 μm, 120 μm, 130 μm, 140 μm, 150 μm, 160 μm, 170 μm, 180 μm, 190 μm, 200 μm, 220 μm, 240 μm, 260 μm, 280 μm, 300 μm, 320 μm, 340 μm, 360 μm, 380 μm, 400 μm, 450 μm, 500 μm, 550 μm, 600 μm, 650 μm, 700 μm, 750 μm, 800 μm, 850 μm, 900 μm, 950 μm or more in thickness suitable for use in testing or clinical applications where such thicknesses are useful.

a. Mesenchymal Stem cell (MSC)-Derived Bioengineered Constructs

Mesenchymal stem cells (MSCs; alternatively known as mesenchymal progenitor cells) are cells capable of expanding in culture and differentiating into mesenchymal tissue cells, including bone, cartilage, tendon, ligament, muscle, adipose, and marrow stroma. MSCs inefficiently synthesize, secrete, and/or organize extracellular matrix components

(*i.e.*, endogenous extracellular matrix production) under normal culture conditions. However, under culture conditions described further herein, they can contain themselves within an efficiently secreted extracellular matrix without exogenous matrix components (*i.e.*, matrix components not produced by the cultured cells but introduced by other means).

MSCs can be obtained from a number of sources including, but not limited to, bone marrow, umbilical cord, placenta, amnion and other connective tissues (e.g. muscle, adipose, bone, tendon and cartilage). For example, umbilical cord MSC's can be isolated from umbilical cord blood, umbilical vein subendothelium, and the Wharton's Jelly. MCSs can further be isolated from three regions: the perivascular zone (umbilical cord perivascular cells or UCPVCs), the intervascular zone, placenta, amnion, and the subamnion (Troyer and Weiss, 2007). Alternatively, bone marrow-derived MSC's can be harvested from bone marrow and comprise non-hematopoietic, multipotential cells, support hematopoietic stem cell expansion, and can differentiate into various connective tissues.

Human cells, as well as those from other mammalian species including, but not limited to, equine, canine, porcine, bovine, ovine, or rodent (*e.g.*, mouse or rat) can be used. The cells can be derived as primary cells from relevant tissues or more preferably from serially passaged or subcultured from established cell stocks or banks that have been screened against viral and bacterial contamination and tested for purity. In addition, cells that are spontaneously, chemically or virally transfected or recombinant cells or genetically engineered cells can also be used in this invention. Also, the cells can be recombinant or genetically-engineered. For example, the cells can be engineered to produce and deliver recombinant cell products such as growth factors, hormones, peptides or proteins, to a subject for a continuous amount of time or as needed when biologically, chemically, or thermally signaled due to the conditions present in the subject. Either long- or short-term gene product expression can be engineered. Long term expression is desirable when the cultured tissue construct is implanted or applied to a subject to deliver therapeutic products to the subject for an extended period of time. Conversely, short term expression is desired in instances where once a wound has healed, the gene products from the cultured tissue construct are no longer needed or can no longer be desired at the site. Cells can also be genetically engineered to express proteins or different types of extracellular matrix components which are either 'normal' but expressed at high levels or modified in some way to make a bioengineered complex comprising extracellular matrix and living cells that is

therapeutically advantageous for improved wound healing, facilitated or directed neovascularization, or minimized scar or keloid formation.

In order to efficiently secrete extracellular matrix to a desired thickness, MSCs can be cultured for a number of days or weeks (*e.g.*, 18, 19, 20, 21, 22, 23, 24, 25 or longer days) in an undefined medium or a chemically defined medium. In a chemically defined system comprising human-derived cells but no chemically undefined or non-human biological components or cells can be used. The cultures are maintained in an incubator to ensure sufficient environmental conditions of controlled temperature, humidity, and gas mixture for the culture of cells according to well known environmental variables. For example, the incubator can be between about 34 °C to about 38 °C (*e.g.*, 37 ± 1 °C) with an atmosphere between about 5-10 ± 1% CO_2 and a relative humidity (Rh) between about 80-90%. Alternatively, cells can be cultured under hypoxic conditions. The cells can be temporarily exposed to ambient room temperature, air, and humidity during feeding, seeding , or other cell manipulations.

Regardless of cell type, culture media is comprised of a nutrient base usually further supplemented with other components. Nutrient bases, which generally supply such nutrients as glucose, inorganic salts, an energy source, amino acids, and vitamins, are well known in the art of animal cell culture. Examples include, but are not limited to, Dulbecco's Modified Eagle's Medium (DMEM); Minimal Essential Medium (MEM); M199; RPMI 1640; Iscove's Modified Dulbecco's Medium (EDMEM). Minimal Essential Medium (MEM) and M199 require additional supplementation with phospholipid precursors and non-essential amino acids. Commercially available vitamin-rich mixtures that supply additional amino acids, nucleic acids, enzyme cofactors, phospholipid precursors, and inorganic salts include Ham's F-12, Ham's F-10, NCTC 109, and NCTC 135. Mixtures of such media can also be used, such as DMEM and Ham's F-12 between a 3-to-1 ratio to a 1-to-3 ratio, respectively.

Culture media formulations and additional dosing with media supplements for MSCs and additional cell types, such as fibroblasts or epithelial cells, can be selected according to well known cell culture methods in the art (see, for example, U.S. Patent No. 5,712,163 to Parenteau, PCT Publication No. WO 95/31473, PCT Publication No. WO 00/29553, PCT Publication No. WO 2009/070720, Ham and McKeehan, Methods in Enzymology, 58:44-93 (1979), Bottenstein et al., Meth. Enzym., 58:94-109 (1979); each of which is incorporated herein in its entirety by this reference). For example, MSC-derived

bioengineered constructs can be cultured in media supplemented with agents that promote matrix synthesis and deposition by the cells. Chemically defined culture media can be used that is free of undefined animal organ or tissue extracts such as serum, pituitary extract, hypothalamic extract, placental extract, or embryonic extract or proteins and factors secreted by feeder cells. Such media can be free of undefined components and biological components derived from non-human animal sources to diminish the risk of adventitious animal or cross-species virus contamination and infection. Synthetic or recombinant functional equivalents can replace the use of such animal organ or tissue extracts.

Transforming growth factor alpha (TGF-α), which is produced in macrophages, brain cells, and keratinocytes, and induces epithelial development, has been found herein to stimulate MSCs to synthesize, secrete, and organize extracellular matrix components to an appreciable degree. TGF-α is a small (~50 residue) protein that shares 30% structural homology with EGF and competes for the same surface-bound receptor site. It has been implicated in wound healing and promotes phenotypic changes in certain cells. TGF-α or long-chain TGF-α can be supplemented to the medium in the range from about 0.0005 μg/mL to about 0.30 μg/mL, from about 0.0050 μg/mL to about 0.03 μg/mL, or from about 0.01 μg/mL to about 0.02 μg/mL. In some embodiments, the amount of supplemented TGF alpha is 10 ng/mL, 20 ng/mL, 30 ng/mL, 40 ng/mL, 50 ng/mL, 60 ng/mL, 70 ng/mL, 80 ng/mL, 90 ng/mL, 100 ng/mL, 120 ng/mL, 130 ng/mL, 140 ng/mL, 150 ng/mL, 160 ng/mL, 170 ng/mL, 180 ng/mL, 190 ng/mL, 200 ng/mL or more.

By contrast, prostaglandin E_2 (PGE_2) is generated from the action of prostaglandin E synthases on prostaglandin H_2 (PGH_2) and has been found herein to inhibit MSCs from synthesizing, secreting, and organizing extracellular matrices when present in relatively high doses. Thus, PGE_2 (*e.g.*, the 16,16 PGE_2 form) supplementation can be used to regulate extracellular matrix thickness and can range from about 0.000038 μg/mL to about 0.760 μg/mL, from about 0.00038 μg/mL to about 0.076 μg/mL, or about 0.038 μg/mL. In some embodiments, the amount of supplemented PGE_2 is 10 ng/mL, 20 ng/mL, 30 ng/mL, 40 ng/mL, 50 ng/mL, 60 ng/mL, 70 ng/mL, 80 ng/mL, 90 ng/mL, 100 ng/mL, 120 ng/mL, 130 ng/mL, 140 ng/mL, 150 ng/mL, 160 ng/mL, 170 ng/mL, 180 ng/mL, 190 ng/mL, 200 ng/mL or more.

Similarly, basic fibroblast growth factor (bFGF) has been found herein to inhibit cells, such as fibroblasts, from synthesizing, secreting, and organizing extracellular matrix components. In particular, pepsin-soluble collagen, sulfated glycosaminoglycans (sGAGs)

and hyaluronic acid (A) are reduced as bFGF levels increase and each component can be reduced by 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or more relative to a control. Such differences in extracellular matrix component composition further result in a powdered form upon air drying and easily ground powder when lyophilized. Such powdered forms have reduced viscosity such that they can pass through syringe needles having a gauge of 23, 24, 25, 26, 27, 28, 29, 30, or finer. Thus, bFGF supplementation can be used to regulate extracellular matrix thickness and composition from about 10 ng/mL, 15 ng/mL, 20 ng/mL, 25 ng/mL, 30 ng/mL, 35 ng/mL, 40 ng/mL, 45 ng/mL, 50 ng/mL, 55 ng/mL, 60 ng/mL, 65 ng/mL, 70 ng/mL, 75 ng/mL, 80 ng/mL, 85 ng/mL, 90 ng/mL, 95 ng/mL, 100 ng/mL or more.

Ascorbate or a derivative (e.g., sodium ascorbate, ascorbic acid, or one of its more chemically stable derivatives such as L-ascorbic acid phosphate magnesium salt n-hydrate) can be used as a supplement to promote proline hydroxylation and secretion of procollagen, a soluble precursor to deposited collagen molecules. Ascorbate also upregulates type I and type III collagen synthesis.

Insulin can be used as a supplement to promote the uptake of glucose and amino acids to provide long term benefits over multiple passages. Supplementation of insulin or insulin-like growth factor (IGF) is necessary for long term culture as there will be eventual depletion of the cells' ability to uptake glucose and amino acids and possible degradation of the cell phenotype. Insulin can be derived from either animal, for example bovine, human sources, or by recombinant means as human recombinant insulin. Therefore, a human insulin would qualify as a chemically defined component not derived from a non-human biological source. Insulin supplementation is advisable for serial cultivation and is provided to the media at a wide range of concentrations. A preferred concentration range is between about 0.1 μg/ml to about 500 μg/ml, at about 5 μg/ml to about 400 μg/ml, and at about 375 μg/ml. Appropriate concentrations for the supplementation of insulin-like growth factor, such as IGF-1 IGF-2, and the like can be easily determined by one of skill in the art for the cell types chosen for culture.

Transferrin can be used as a supplement to regulate iron transport. Iron is an essential trace element found in serum but can be toxic in large amounts if not sequestered by transferrin. Transferrin can be supplemented at a concentration range of between about 0.05 to about 50 μg/ml or at about 5 μg/ml.

Triiodothyronine (T3) can be used as a supplement to regulate cell metabolism and can be supplemented at a concentration range between about 0 to about 400 ρM, between about 2 to about 200 ρM, or at about 20 ρM.

Either or both ethanolamine and o-phosphoryl-ethanolamine, which are phospholipids, can be used as a supplement to facilitate fatty acid production, particularly when culturing in a serum-free medium. Ethanolamine and o-phosphoryl-ethanolamine can be supplemented at a concentration range between about 10^{-6} to about 10^{-2} M or at about 1 x 10^{-4} M.

Selenious acid can be used as a supplement to provide the trace element in serum-free media. Selenious acid can be provided at a concentration range of about 10^{-9} M to about 10^{-7} M or at about 5.3 x 10^{-8} M.

Supplementation with amino acids can conserve cellular energy by bypassing the cell's need to synthesize these building blocks of proteins. For example, the addition of proline and glycine, as well as the hydroxylated form of proline, hydroxyproline, are basic amino acids that make up the structure of collagen. In addition, the amino acid L-glutamine is present in some nutrient bases and can be added in cases where there is none or insufficient amounts present. L-glutamine can also be provided in stable form such as that sold under the mark, GlutaMAX-1™ (Gibco BRL, Grand Island, NY). GlutaMAX-1™ is the stable dipeptide form of L-alanyl-L-glutamine and can be used interchangeably with L-glutamine and is provided in equimolar concentrations as a substitute to L-glutamine. The dipeptide provides stability to L-glutamine from degradation over time in storage and during incubation that can lead to uncertainty in the effective concentration of L-glutamine in medium. Typically, the base medium is supplemented with preferably between about 1 mM to about 6 mM, more preferably between about 2 mM to about 5 mM, and most preferably 4 mM L-glutamine or GlutaMAX-1™.

Additional supplements can also be added for particular culture outcomes, such as one or more prostaglandins, transforming growth factors (including transforming growth factors alpha or beta), keratinocyte growth factor (KGF), connective tissue growth factor (CTGF), or mannose-6-phosphate (M6P), or combinations thereof. For example, TGF-ß1 and TPA are each known to upregulate collagen synthesis (Raghow et al., J. Clin. Invest., 79:1285-1288 (1987) and Pardes et al., J. Invest. Derm., 100:549 (1993)).

In addition, epidermal growth factor (EGF) can be used as a supplement to help establish cultures through cell scale-up and seeding. EGF in native form or recombinant

form can be used. Human forms, native or recombinant, of EGF are preferred for use in the medium when fabricating a skin equivalent containing no non-human biological components. EGF is an optional component and can be provided at a concentration between about 1 to 15 ng/mL or between about 5 to 10 ng/mL.

Hydrocortisone can be used as a supplement to promote keratinocyte phenotype and therefore enhance differentiated characteristics such as involucrin and keratinocyte transglutaminase content (Rubin et al., J. Cell Physiol., 138:208-214 (1986)). Therefore, hydrocortisone is a desirable additive in instances where these characteristics are beneficial such as in the formation of keratinocyte sheet grafts or skin constructs. Hydrocortisone can be provided at a concentration range of about 0.01 μg/ml to about 4.0 μg/ml or between about 0.4 μg/ml to 16 μg/ml.

Keratinocyte growth factor (KGF) can be used as a supplement to support epidermalization in the range from about 0.001 μg/mL to about 0.150 μg/mL, from about 0.0025 μg/mL to about 0.100 μg/mL, from about 0.005 μg/mL to about 0.015 μg/mL, or 5 μg/mL.

Mannose-6-phosphate (M6P) can be used as a supplement to support epidermalization at about 0.0005 mg/mL to about 0.0500 mg/mL.

Neutral polymers can be used can be used as a supplement to enhance consistency of collagen processing and deposition between samples. For example, polyethylene glycol (PEG) is known to promote *in vitro* processing of the soluble precursor procollagen produced by the cultured cells to a matrix-deposited collagen form. Tissue culture grade PEG within the range between about 1000 to about 4000 MW (molecular weight), about 3400 to about 3700 MW, at about 5% w/v or less, about 0.01% w/v to about 0.5% w/v, about 0.025% w/v to about 0.2% w/v, or about 0.05% w/v. Other culture grade neutral polymers such dextran, preferably dextran T-40, or polyvinylpyrrolidone (PVP), preferably in the range of 30,000-40,000 MW, can also be used at concentrations at about 5% w/v or less, between about 0.01% w/v to about 0.5% w/v, between about 0.025% w/v to about 0.2% w/v, or about 0.05% w/v. Other cell culture grade and cell-compatible agents that enhance collagen processing and deposition are well known to the skilled artisan.

b. Culture Substrates and/or Perfusion

Seeding the cells on a porous membrane (*i.e.*, culture insert) of a defined diameter can enhance bioengineered construct thicknesses by enhancing the rate at which extracellular matrices are produced, since it maximizes surface area exposure to the media's

nutrients. The pores communicate through both top and bottom surfaces of the membrane to allow bilateral contact of the medium to the developing tissue construct or for contact from only below the culture. Media can also contact only the bottom of the forming cultured tissue construct, so that the top surface can be exposed to air, as in the development of a cultured skin construct. Typically, the membrane is secured to one end of a tubular member or framework that is inserted within and interfaces with a base, such as a petri or culture dish that can be covered with a lid. When these types of culture vessels are employed, the tissue-construct is produced on one surface of the membrane (*e.g.*, the top, upwardly facing surface) and the culture is contacted by cell media on both top and bottom surfaces. The pore sizes are small enough that it does not allow for the growth of cells through the membrane, yet large enough to allow for free passage of nutrients contained in culture medium to the bottom surface of the bioengineered construct, such as by capillary action. For example, the pore sizes can be about less than 7 μm, between about 0.1 μm to about 7 μm, between about 0.2 μm to about 6 μm, or between about 0.4 μm to about 5 μm in diameter. The maximum pore size depends not only on the size of the cell but also the ability of the cell to alter its shape and pass through the membrane. It is important that the tissue-like construct adheres to the surface but does not incorporate or envelop the substrate so it is removable from it such as by peeling with minimal force. The size and shape of the tissue construct formed is dictated by the size of the vessel surface or membrane on which it is grown. Substrates can be round, square, rectangular or angular or shaped with rounded corner angles, or irregularly shaped. Substrates can also be flat or contoured as a mold to produce a shaped construct to interface with a wound or mimic the physical structure of native tissue. To account for greater surface areas of the growth substrate, proportionally more cells are seeded to the surface and a greater volume of media is needed to sufficiently bathe and nourish the cells. When the bioengineered-based tissue construct is finally formed, it is removed by peeling from the membrane substrate. Substrates can be pre-treated prior to cell seeding in order to improve bonding characteristics of the substrate by raising the surface energy. Pre-treatment can include, but is not limited to, COOH and Long NH_2 treatment.

Perfusing the culture substrate to exert a mechanical force against the forming bioengineered layer to mimic *in vivo* forces can further enhance bioengineeered construct thickness and strength. Perfusion means are well known in the art and include, but are not limited to, stirring the media using a magnetic stir bar or motorized impeller subjacent or

adjacent to the substrate carrier containing the culture membrane; pumping media within or through the culture dish or chamber; gently agitating the culture dish on a shaking or rotating platform; or rolling if a roller culture bottle is used. Other mechanical forces can be exerted by pulsing, flexing, undulating or stretching of the porous membrane during culture.

During the culture, cells secrete endogenous matrix molecules and organize the secreted matrix molecules to form a three dimensional tissue-like structure but do not exhibit significant contractile forces to cause the forming bioengineered construct to contract and peel itself from the culture substrate. Suitable cell growth surfaces on which the cells can be grown can be any biologically compatible material to which the cells can adhere and provide an anchoring means for the bioengineered construct to form. Materials such as glass; stainless steel; polymers, including polycarbonate, poly(ether sulfones) (PES), polystyrene, polyvinyl chloride, polyvinylidene, polydimethylsiloxane, fluoropolymers, and fluorinated ethylene propylene; and silicon substrates, including fused silica, polysilicon, or silicon crystals can be used as a cell growth surface. The cell growth surface material can be chemically treated or modified, electrostatically charged, or coated with biologicals such as poly-l-lysine or peptides. An example of a chemical treatment that results in an electrostatically charged surface COOH and Long NH_2. An example of a peptide coating is RGD peptide. The cell growth surface can be treated with a synthetic or human form of extracellular matrix that assists with the attachment of the matrix producing cells so that the cells have a natural interface with the cell growth surface for attachment, orientation, and biochemical cues. When a synthetic or human form of extracellular matrix is used in this aspect, it is temporary because it is replaced by the cells over time in culture. The synthetic or human form of extracellular matrix, when deposited on the cell-growth surface range from matrix molecules dispersed across the surface, to molecular thickness, or to continuous thin film of between nanometer to micrometer thickness.

Fibronectin in natural and synthetic forms can be used to provide a coating to the culture substrate. The fibronectin forms which can be used, include but are not limited to: human fibronectin, human plasma-derived fibronectin, recombinant fibronectin, or synthetic forms such as ProNectin, which is a repeated peptide sequence derived and synthesized from a portion of natural human fibronectin. Coatings of natural, cell-culture produced or recombinant collagen can be provided to the substrate.

The cultured bioengineered constructs do not rely on synthetic or bioresorbable members, such as a mesh member, for formation and integrity; however, such members can be used. A mesh member can be a woven, knit, or felt like material. In systems where a mesh member is used, the cells are cultured on the mesh member and grow on either side and within the interstices of the mesh to envelop and incorporate the mesh within the cultured tissue construct. The final construct formed by methods that incorporate such a mesh rely on it for physical support and for bulk.

Silk scaffolds can provide structural support, while illiciting minimal or no host immune response. The porosity of the porous silk fibroin scaffold can range from between about 10 microns to about 150 microns, 30 microns to about 45 microns, 50 microns to 100 microns, or 80 microns to 150 microns in diameter.

Average pore diameter of the silk scaffolds can be controlled by varying the solvent percentage. Silk fibers can be mixed with an organic solvent, such as ethanol or DMSO. By increasing the amount of organic solvent, the pore size of the silk scaffolds can be selectively decreased based upon a desired level of porosity. For example, dissolving 4% silk to 1% ethanol results in a silk scaffold that has an average pore diameter of 50-100 microns. A pore size between 50 and 100 microns is desirable for enhanced fibroblast infiltration and for allowing faster vascularization of the construct *in vivo*. A larger silk scaffold average pore diameter (*e.g.,* about 80-150 microns) can be achieved by dissolving 3% silk into 0.5% ethanol. A silk scaffold with an average pore diameter of about 80-150 microns is desirable for more severe burn wounds because the larger pores allows for wound exudates to be cleared from the wound bed.

Silk fibroin can be derived from either natural or recombinant sources. A preferred natural source of silk fibroin is derived from the degummed silk fiber of a Bombyx Mori silkworm cocoon. A solution of silk fibroin is admixed with a water-miscible organic solvent such as an alcohol selected from the group consisting of ethyl alcohol, methyl alcohol, isopropyl alcohol, propanol, butanol; or dimethylsulfoxide (DMSO) or acetone. The silk fibroin solution is then cast or poured into a mold or directly into a culture insert that incorporates a porous/permeable culture membrane that provides for bilateral contact of culture medium both above and below the planar surface of the membrane and porous silk fibroin scaffold. The solution is then frozen for a time, then thawed and rinsed to remove solvent residues. The porous silk fibroin scaffolds are then autoclaved, gamma irradiated or e-beam sterilized to produce a sterile porous silk fibroin scaffold. After

sterilization, the porous silk fibroin scaffold can be used as a culture substrate for cultured cells using the methods employed herein. After culturing cells on the porous silk fibroin scaffolds, the cells can also be devitalized using the methods employed herein. Other features can be added to the porous silk fibroin scaffold constructs, such as a silicone layer.

Silk scaffolds can be conditioned with substances useful for enhancing wound healing. For example, wet or dry silk scaffolds can be incubated with a solution containing one or more proteins for 5-10 minutes such that the final amount of protein adsorbed is in the range of 1 microgram to 1 milligram. Silk scaffolds and bioengineered constructs comprising silk scaffolds that are partially lyophilized (*e.g.*, freeze dried for 3 hours at 0°C) and frozen at -20°C prior to incubation with protein solutions appear to maximize the amount of adsorbed protein. Autoclaving the silk scaffold prior to use in cell culture also appears to enhance *in vivo* degradation and thus reduced persistence.

c. Cell Seeding

Seeding at superconfluency (*i.e.*, greater than 100% confluency) increases the rate of extracellular matrix formation by bypassing the cellular growth phase. Thus, cells can be directly seeded at superconfluence from 100% confluence up to about 900% confluence, including in the range of about 300% to about 600% confluence to immediately produce an extracellular matrix. Superconfluency can also be achieved according to cell seeding densities per culture surface area and can be, for example, 1×10^5, 2×10^5, 3×10^5, 4×10^5, 5×10^5, 6×10^5, 7×10^5, 8×10^5, 9×10^5, 1×10^6 or more cells per cm^2. For example, 75 mm diameter inserts can be used which have an approximate culture surface area of 44 cm^2. Seeding a superconfluent number of cells (*e.g.*, 3×10^6 cells) on such an insert results in an initial seeding density of about 6.8×10^5 cells/cm^2. Approximately 7.5×10^6 cells can be seeded onto a 10 cm x 10 cm rectangular insert to produce an initial seeding density of about 7.5×10^5 cells/cm^2.

Alternatively, cells can be seeded at sub-confluence to proliferate prior to stimulating them to produce and organize an extracellular matrix. Sub-confluent cell density can be achieved by seeding between about 1×10^5 cells/cm^2 to about 6.8×10^5 cells/cm^2, between about 3×10^5 cells/cm^2 to about 6.8×10^5 cells/cm^2, or about 6.8×10^5 cells/cm^2 (cells per square centimeter area of the surface).

d. Controlled Contraction

The thickness of a bioengineered construct can be enhanced by releasing it from the

culture substrate, so that it is allowed to contract without restraint. Such "controlled contraction" or "unrestrained contraction" can be monitored in real time and can be stopped after a desired amount of contraction and thickness has occurred. The living cells in the bioengineered construct exert contractile forces on the endogenous extracellular matrix that are mitigated by adherence of the bioengineered construct to the culture substrate. In the unrestrained contraction step, these contractile forces imparted by the cells are leveraged to increase the overall physical strength and thickness of the construct as compared to similarly prepared constructs that have not been subjected to unrestrained contraction after culturing. Controlled contraction can be induced by releasing the bioengineered construct from the culture substrate such as by using physical means such as by peeling or lifting it from the substrate, shaking it from the substrate, or by flexing the substrate. Release of the bioengineered construct can also be achieved by changing the temperature of the culture, especially when a thermoresponsive substrate is employed, or by using chemical means.

Controlled contraction is measured by time, by increased thickness, and by a decrease in surface area, as measured by the decrease in diameter or decrease of the width and length, of the construct. Contraction of the matrix by the cells appears to organize the fibers of the endogenous matrix such that they increase the overall strength of the matrix (*e.g.*, suture retention strength), but not so much that the matrix becomes misshapen, distorted, wrinkled or loses an approximate planarity in its configuration. In other words, the flat planar aspect of the matrix is preserved, but the overall surface area decreases and thickness increases. When unrestrained contraction is measured by the overall increase of the bioengineered thickness, a percentage thickness increase or an actual increased thickness measure is used. When unrestrained contraction is measured by decrease in surface area, a percentage decrease in the surface area or an actual measure of decrease of one or more dimensions is used. Contraction can be measured by measuring the percentage decrease in the surface area of the tissue matrix, such as between 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or more or any range in between. Contraction can be stopped, when appropriate, by devitalizing the cells such as described further herein.

e. Hybrid Bioengineered Constructs

MSC-derived bioengineered constructs can further comprise additional cell types capable of synthesizing, secreting, and organizing extracellular matrix to enhance extracellular matrix thicknesses. Such cell types can be fibroblasts, stromal cells, smooth muscle cells, chondrocytes and other connective tissue cells of mesenchymal origin.

Fibroblast cells can be derived from a number of sources, including, but not limited to neonate male foreskin, dermis, tendon, lung, umbilical cords, cartilage, urethra, corneal stroma, oral mucosa, and intestine. Chimeric mixtures of normal cells from two or more sources, such as a chimeric mixture of autologous and allogeneic cells; mixtures of normal and genetically modified or transfected cells; mixtures of cells derived from different tissue or organ types; or, mixtures of cells of two or more species or tissue sources can be used.

The at least one additional cell type can be added in layered or admixed form. For layered bioengineered constructs, a first cell type is seeded upon a cell culture substrate and a second cell type is subsequently seeded atop the first layer of cells. Admixed constructs can be generated by varying the initial seeding ratios of the at least two cells types based at least in part upon desired construct attributes for therapeutic effect. For example, MSCs can be the first cell type and comprise 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or more of the initial cell seeding mixture. Fibroblasts, such as neonatal fibroblasts, dermal fibroblasts, papillary fibroblasts, reticular fibroblasts, or a combination thereof, can be the second cell type and comprise the remaining initial cell seed mixture. The total cell population at initial seeding can be between 1.0×10^5 to 1.0×10^6 per cm^2.

For bioengineered constructs produced by admixing, initial seeding densities can also be determined based on the number of cells at the time of seeding, where the total cell mass desired is known at the time of seeding according to: $aX + bY = Z$; wherein $X = Y = Z$ and $a + b = 1$, but $b > 0$ and $a < 1$. For example, the desired cell seeding density is Z and $Z = 2.1 \times 10^5$ cells/cm^2 (approximately) and aX and bY represent the number of fibroblasts and mesenchymal progenitor cells, respectively, in the total number of cells per square centimeter of area to be seeded represented by Z. Thus, where fibroblasts and MSCs each comprise 50% of the total cells seeded, the equation would be expressed as: $aX + bY = Z$ cells/cm^2 where $(0.5)(2.1 \times 10^5$ cells$) + (0.5)(2.1 \times 10^5$ cells$) = 2.1 \times 10^5$ total cells/cm^2. Solving this equation leads to determining the initial seeding density of both of the at least two cell types: 1.05×10^5 fibroblasts + 1.05×10^5 mesenchymal progenitor cells = 2.1×10^5 total cells/cm^2. When this seeding equation is employed, the following can be used: $a = 0$ and $b = 1$; $a = 0.1$ and $b = 0.9$; $a = 0.2$ and $b = 0.8$; $a = 0.3$ and $b = 0.7$; $a = 0.5$ and $b = 0.5$; $a = 0.8$ and $b = 0.2$.

Alternatively, hybrid bioengineered constructs can be produced by fibroblasts and MSCs, wherein X is constant (i.e., the number of fibroblasts is kept constant) where total

number of fibroblasts in the total cell mass is known at the time of seeding according to: aX + bY = Z; wherein X = Y, a = 1, b > 0 and b < 1, and Z = the calculated seeding density of the total cell mass. For example, if X = 2.1 x 10^5 fibroblasts and 50% MSCs is desired at seeding, the equation would be expressed as: aX + bY = Z, where (1)(2.1 x 10^5 cells) + (0.5)(2.1 x 10^5 cells) = Z total cells/cm^2. Solving this equation leads to determining the initial seeding density of both of the at least two cell types: 2.1 x 10^5 fibroblasts + 1.05 x 10^5 mesenchymal progenitor cells = 3.15 x 10^5 total cells/cm^2. When this seeding equation is employed, the following can be used: a = 1 and b= 2; a = 1 and b= 1; a = 1 and b = 0.9; a = 1 and b = 0.8; a = 1 and b = 0.7; a = 1 and b = 0.5; a = 1 and b = 0.2.

II. Controlling Bioengineered Construct Pore Size

Certain constructs may be porous in structure. Porosity may be measured by the surface area attributed to pores in a histology image relative to the total surface area of the image. Certain constructs can have a porosity of at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or more.

The average pore size within the extracellular matrix of bioengineered constructs can be engineered to form porous extracellular matrix and/or regulate pore size. Combined with a type and/or degree of crosslinking, defined average pore sizes can be chosen and controlled to yield constructs that have different rates of *in vivo* persistence and/or cell infiltration, ranging from "rapidly bioremodelable" to "moderately bioremodelable" to "prolonged bioremodelable" bioengineered constructs for tailored applicability to therapeutic uses. In addition, smaller pore sizes can be engineered to enhance barrier functions where prevention or inhibition of cell infiltration, such as undesirable host cell types, is useful.

Average pore size (diameter) can be engineered by varying the final temperature at which lyophilization, also known as freeze-drying, occurs. In this process, the bioengineered constructs are frozen such that the aqueous aspects of the bioengineered construct achieve a frozen state, after which, the bioengineered construct is subjected to a vacuum to remove the frozen water (ice) from the construct. Lyophilization creates and opens up the pore structure by removing the ice crystals that form in the matrix and the freezing temperature determines the resulting average pore size. Thus, performing lyophilization at colder freezing temperatures generates smaller pore sizes, whereas performing lyophilization at warmer freezing temperatures generates larger pore sizes. Thus, in one embodiment, the temperature can range between -100°C and 0°C with an

average pore size of less than 5 to 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more microns (um) in size as the freezing temperature warms. In one embodiment, average pore size of less than 5, 10, 15, 20, 25, or 30 um in size or any range in between can be produced at a freezing temperature of -40°C. In another embodiment, average pore sizes of at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more um in size or any range in between can be produced at a freezing temperature of -10°C. Decreasing the rate toward reaching the freezing temperature can increase the uniformity of pore size. Thus, decreasing the rate to freezing by 10, 5, 4, 3, 2, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.3, 0.1, or fewer °C per minute, or any range in between, can increase the uniformity of the pores in the construct..

III. Controlling Bioengineered Construct Composition

The extracellular matrices of the bioengineered constructs of the present invention comprise components useful for treating and healing wounds.

a. Devitalized Bioengineered Constructs

The bioengineered constructs of the present invention can be devitalized, to terminate the cells without removal, and/or decellularized to remove the cells, depending upon their ultimate use in treating a subject. Devitalization or decellularization can occur either on the membrane of the culture insert or after the bioengineered construct is removed from the culture insert.

Bioengineered constructs can be devitalized in a number of ways. One method for devitalizing the cells in the bioengineered construct is to remove all or substantially all of the moisture in the construct using physical means. Means for removing moisture include dehydration in air, by freezing or by freeze-drying. To dehydrate the construct by air-drying, culture medium is removed from the vessel in which the bioengineered construct is made and the bioengineered construct is simply allowed to dehydrate for a sufficient time to allow the cells to die. Dehydration conditions vary in terms of temperature and relative humidity. Dehydration temperatures can range from above freezing temperature up to the denaturation temperature of the collagen (as measured by differential scanning calorimetry, or "DSC") in the bioengineered construct, for example, between about 0°C to about 60°C or ambient room temperature (*e.g.*, about 18°C to about 22°C). Relative humidity values that are lower, as in the range of about 0% to about 60%, are preferred; however, relative humidities comparative to room humidity, between about 10% Rh to about 40% Rh are

also preferred. When dehydration is conducted by air-drying at ambient room temperature and humidity, the bioengineered construct will have about 10% to about 40% w/w moisture, or less. Alternatively, the bioengineered construct can be freeze-dried (*i.e.*, lyophilized), wherein the construct is frozen and then placed in a vacuum environment to remove the moisture. For example, bioengineered constructs can be taken straight out of culture and frozen (e.g., at a temperature between -80°C to 0°C or any range in between), and lyophilized overnight, such as between about 1 to about 15 hours, or longer. Alternatively, bioengineered constructs can first be air-dried for about eight hours and then subsequently frozen and lyophilized. After drying in ambient conditions or by freeze-drying, the bioengineered construct is devitalized but still retains devitalized cells and cell remnants. Lyophilization can also impart qualities different than those that can result when dehydrating under ambient conditions. Such qualities, in one embodiment, exhibits a more porous and open fibrous matrix structure.

Chemical means can also be employed to devitalize the cells in the bioengineered construct. Water to osmotically terminate the cells can be used. Bioengineered constructs can be immersed in sterile, pure water for a time sufficient to allow for hypotonic swelling to cause the cells to lyse. After the cells lyse, the bioengineered construct can be devitalized but still retain devitalized cells and cell remnants. When water is used, it can also be mixed with other substances, such as peracetic acid or hydrogen peroxide, or salts, or a combination thereof. For example, a devitalizing solution of peracetic acid between about 0.05% to about 3% v/v in water can be used. This devitalizing agent can also be buffered or contain a high salt concentration to prevent excessive swelling of the bioengineered construct when terminating the cells. Alternatively, organic solvents and organic solvent solutions can be used as devitalizing agents in the invention. Organic solvents are capable of displacing the water in a bioengineered construct to terminate, therefore, devitalizing the cells in the bioengineered construct. The organic solvent used to remove water can be one that leaves no residues when removed from the construct that include, but are not limited to, alcohols (*e.g.*, ethyl alcohol, methyl alcohol and isopropyl alcohol) and acetone. For example, bioengineered constructs can be immersed in sterile ethyl alcohol for a time sufficient to displace water in the bioengineered construct and devitalize the cells. The ethyl alcohol can be removed before exposure to air for a time sufficient to allow the absorbed ethyl alcohol in the bioengineered construct to evaporate.

After evaporation of solvent, the construct retains the devitalized cells and cell remnants and is dehydrated.

Other means to devitalize the cells include subjecting the bioengineered constructs to ultraviolet light or gamma irradiation. These means can be performed in conjunction with hypotonic swelling with water, or other chemical devitalizing means or with air and freezing.

b. Decellularized Bioengineered Constructs

Decellularization results in removal of the extracellulmatrix-producing cells that generate the endogenous extracellular matrix components of the bioengineered constructs from the completed construct. One method for decellularizing uses immersion or gentle agitation within a series of chemical treatments to remove the cells, cell remnants, and residual cellular DNA and RNA. Other non-collagenous and non-elastinous extracellular matrix components can also be removed or reduced with the agents and methods used to decellularize, such as glycoproteins, glycosaminoglycans, proteoglycans, lipids, and other non-collagenous proteins present in the ECM. For example, the bioengineered construct can be first treated by contacting it with an effective amount of chelating agent, preferably physiologically alkaline to controllably limit swelling of the cell- matrix. Chelating agents enhance removal of cells, cell debris and basement membrane structures from the matrix by reducing divalent cation concentration. Alkaline treatment can dissociate glycoproteins and glycosaminoglycans from the collagenous tissue and saponify lipids. Chelating agents known in the art which can be used include, but are not limited to, ethylenediaminetetraacetic acid (EDTA) and ethylenebis(oxyethylenitrilo)tetraacetic acid (EGTA). EDTA is can be made more alkaline by the addition of sodium hydroxide (NaOH), calcium hydroxide $Ca(OH)_2$, sodium carbonate or sodium peroxide. EDTA or EGTA concentrations can be between about 1 to about 200 mM, between about 50 to about 150 mM, or about 100 mM. NaOH concentration can be between about 0.001 to about 1 M, between about 0.001 to about 0.10 M, or about 0.01 M (*e.g.*, 100 mM EDTA/10 mM NaOH in water). Other alkaline or basic agents can be determined by one of skill in the art to bring the pH of the chelating solution within the effective basic pH range. The final pH of the basic chelating solution should be between about 8 and about 12 or between about 11.1 to about 11.8.

The bioengineered construct can then be contacted with an effective amount of acidic solution optionally containing a salt. Acid treatment can enhance the removal of

glycoproteins, glycosaminoglycans, non-collagenous proteins, and nucleic acids. Salt treatment can control the swelling of the collagenous matrix during acid treatment and enhance the removal of some glycoproteins and proteoglycans from the collagenous matrix. Acid solutions known in the art can be used and can include, but are not limited to, hydrochloric acid (HCl), acetic acid (CH_3COOH) and sulfuric acid (H_2SO_4). For example, hydrochloric acid (HCl) can be used at a concentration between about 0.5 to about 2 M, between about 0.75 to about 1.25 M, or around 1 M. The final pH of the acid/salt solution should be between about 0 to about 1, between about 0 and 0.75, or between about 0.1 to about 0.5. Hydrochloric acid and other strong acids are most effective for breaking up nucleic acid molecules, while weaker acids are less effective. Salts that can be used are preferably inorganic salts and include, but are not limited to, chloride salts such as sodium chloride (NaCl), calcium chloride ($CaCl_2$), and potassium chloride (KCl). For example, chloride salts can be used at a concentration between about 0.1 to about 2 M, between about 0.75 to about 1.25 M, and around 1 M (*e.g.*, 2 M HCl/1 M NaCl in water).

The bioengineered construct can then be contacted with an effective amount of salt solution which is preferably buffered to about a physiological pH. The buffered salt solution neutralizes the material while reducing swelling. Salts that can be used are preferably inorganic salts and include, but are not limited to, chloride salts such as sodium chloride (NaCl), calcium chloride ($CaCl_2$), and potassium chloride (KCl); and nitrogenous salts such as ammonium sulfate (NH_3SO_4). For example, chloride salts can be used at a concentration between about 0.1 to about 2 M, between about 0.75 to about 1.25 M, or about 1 M. Buffering agents are known in the art and include, but are not limited to, phosphate and borate solutions. For example, phosphate buffered saline (PBS) can be used, wherein the phosphate is at a concentration from about 0.001 to about 0.02 M and a salt concentration from about 0.07 to about 0.3 M to the salt solution (*e.g.*, 1 M sodium chloride (NaCl)/10 mM phosphate buffered saline (PBS)). The pH should be between about 5 to about 9, between about 7 to about 8, or between about 7.4 to about 7.6.

After chemical cleaning treatment, the bioengineered construct can then be rinsed free of chemical cleaning agents by contacting it with an effective amount of rinse agent. Agents such as water, isotonic saline solutions (*e.g.*, PBS) and physiological pH buffered solutions can be contacted with the bioengineered construct for a time sufficient to remove the cleaning agents. The cleaning steps of contacting the bioengineered construct with an alkaline chelating agent and contacting the bioengineered construct with an acid solution

containing salt can be performed in either order to achieve substantially the same cleaning effect.

c. Multilayer and/or Crosslinked Bioengineered Constructs

The ECM can be crosslinked using a crosslinking agent to control its rate of bioremodeling and to either increase its persistence when implanted or engrafted into a living body. It can be crosslinked and used as a single layer construct or it can be combined or manipulated to create different types of constructs. Crosslinking can bond bioengineered sheets, or portions thereof, together.

Some bioengineered constructs have two or more superimposed ECM sheets that are bonded together to form a flat-sheet construct. As used herein, "bonded collagen layers" means composed of two or more bioengineered sheets of the same or different origins or profiles treated in a manner such that the layers are superimposed on each other and are sufficiently held together by self-lamination and/or chemical bonding. For example, the bioengineered constructs can comprise any number of layers, such as between 2 and 20 layers or between 2 and 10 layers, with the number of layers depending on the strength and bulk necessary for the final intended use of the construct. Alternatively, as the ultimate size of a superimposed arrangement can be limited by the size of the matrix sheets, the layers can be staggered in a collage arrangement to form a sheet construct with a surface area larger than the dimensions of any individual matrix sheet but without continuous layers across the area of the arrangement.

To form a multilayer bioengineered construct of matrix sheets, a first sterile rigid support member, such as a rigid sheet of polycarbonate, can be laid down. If the matrix sheets are still not in a hydrated state, such as after performance of the devitalizing or decellularizing processes, they are hydrated in aqueous solution, such as water or phosphate buffered saline. Matrix sheets can be blotted with sterile absorbent cloths to absorb excess water from the material. A first matrix sheet can be laid on the polycarbonate sheet and manually smoothed to the polycarbonate sheet to remove any air bubbles, folds, and creases. A second matrix sheet can be laid on the top of the first sheet, again manually removing any air bubbles, folds, and creases. This layering can be repeated until the desired number of layers for a specific application is obtained.

After layering the desired number of matrix sheets, they can then be dehydrated together. Dehydration may bring the extracellular matrix components, such as collagen fibers, in the layers together when water is removed from between the fibers of the adjacent

matrix sheets. The layers can be dehydrated either open-faced on the first support member or, between the first support member and a second support member, such as a second sheet of polycarbonate, placed before drying over the top layer and fastened to the first support member to keep all the layers in flat planar arrangement together with or without compression. To facilitate dehydration, the support member can be porous to allow air and moisture to pass through to the dehydrating layers. The layers can be dried in air, in a vacuum, or by chemical means such as by acetone or an alcohol such as ethyl alcohol or isopropyl alcohol. Dehydration by air-drying can be done to room humidity, between about 0% Rh to about 60% Rh, or less; or about 10% to about 40% w/w moisture, or less. Dehydration can be easily performed by angling the superimposed matrix layers to face a sterile airflow of a laminar flow cabinet for at least about 1 hour up to 24 hours at ambient room temperature, approximately 20°C., and at room humidity. Dehydration conducted by vacuum or chemical means will dehydrate the layers to moisture levels lower than those achieved by air-drying.

In an optional step, the dehydrated layers are rehydrated or, alternatively, rehydrated and dehydrated again. As mentioned above, the dehydration brings the extracellular matrix components of adjacent matrix layers together and crosslinking those layers together forms chemical bonds between the components to bond the layers. To rehydrate the layers, they are peeled off the porous support member together and are rehydrated in an aqueous rehydration agent, preferably water, by transferring them to a container containing aqueous rehydration agent for at least about 10 to about 15 minutes at a temperature between about 4°C to about 20°C to rehydrate the layers without separating or delaminating them. The matrix layers are then crosslinked together by contacting the layered matrix sheets with a crosslinking agent, preferably a chemical crosslinking agent that preserves the bioremodelability of the matrix layers.

Crosslinking provides strength and durability to the construct and improves its handling properties. Various types of crosslinking agents known in the art can be used such as carbodiimides, genipin, transglutaminase, ribose and other sugars, nordihydroguaiaretic acid (NDGA), oxidative agents, ultraviolet (UV) light and dehydrothermal (DHT) methods. Besides chemical crosslinking agents, the layers can be bonded together with biocompatible fibrin-based glues or medical grade adhesives such as polyurethane, vinyl acetate or polyepoxy. One biocompatible adhesive is silk fibroin, that is a 4-8% silk fibroin solution disposed at the bonding region between adjacent layers of tissue matrix that is activated

using methyl alcohol. Biocompatible glues or adhesives can be used to bond crosslinked or uncrosslinked layers, or both, together.

One appropriate crosslinking agent is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). Sulfo-N-hydroxysuccinimide can be added to the EDC crosslinking agent as described by Staros, J.V., Biochem. 21, 3950-3955, 1982. In the most preferred method, EDC is solubilized in water at a concentration between about 0.1 mM to about 100 mM, between about 1.0 mM to about 10 mM, or about 1.0 mM. Besides water, phosphate buffered saline or (2-[N-morpholino]ethanesulfonic acid) (MES) buffer can be used to dissolve the EDC. Other agents can be added to the solution, such as acetone or an alcohol, up to 99% v/v in water and typically 50%, to make crosslinking more uniform and efficient. These agents remove water from the layers to bring the matrix fibers together to promote crosslinking between those fibers. The ratio of these agents to water in the crosslinking agent can be used to regulate crosslinking. EDC crosslinking solution is prepared immediately before use as EDC will lose its activity over time. To contact the crosslinking agent to the matrix layers, the hydrated, bonded matrix layers are transferred to a container such as a shallow pan and the crosslinking agent gently decanted to the pan ensuring that the matrix layers are both covered and free-floating and that no air bubbles are present under or between the matrix layers. The container is covered and the matrix layers are allowed to crosslink for between about 4 to about 24 hours or between 8 to about 16 hours at a temperature between about 4°C to about 20°C. Crosslinking can be regulated with temperature such that at lower temperatures, crosslinking is more effective since the reaction is slowed. By contrast, crosslinking is less effective at higher temperature since the EDC is less stable.

After crosslinking, the crosslinking agent is decanted and disposed of and the crosslinked multi-layer matrix constructs are rinsed by contacting them with a rinse agent (*e.g.*, water) to remove residual crosslinking agent, such as by contacting the crosslinked multi-layer matrix constructs three times with equal volumes of sterile water from anywhere between one minute and forty-five minutes for each rinse.

Alternatively, bioengineered constructs can be crosslinked using dehydrothermal (DHT) crosslinking methods that form covalent bonds between the adjacent carboxy and amino groups on the protein fibers via a condensation reaction when the implants are exposed to controlled heat while under a vacuum (typically 120°C dry heat for up to 24 hrs). In this treatment, water molecules are driven off from the individual fibers often

leading to complex changes in the molecular positioning of amino acids in the collagen chain and possible oxidative damage. DHT can be advantageous over chemical cross-linking for certain regenerative medicine applications since this process does not introduce potentially cytotoxic or inflammatory chemicals into the implants for therapeutic use which would stimulate the patient's immune responses.

DHT has potential to provide high strength to collagen matrixes (~50 MPa), but it is known to partially denature the collagen fibers due to the molecular repositioning of the amino acids within the collagen fibers. The greater number of crosslinks made in a material will typically provide greater durability when the material is exposed to digestive enzymes. However, it is also known that certain protein enzymes only cleave at specific target sites which can not be exposed within triple helical domains of collagen fibers unless and until the protein has been denatured. The level of denaturation that occurs during cross-linking of collagen implantables can be minimized in order to avoid the possible rapid degradation of the matrices by non-specific proteases upon implantation into the patient. Levels of DHT crosslinking in collagenous matrices is typically measured by changes in shrinkage temperature, mechanical loading or sensitivity to enzymatic digestions (*e.g.*, collagenase, trypsin, etc.) of the collagen fibers. The effects of drying and thermal treatment of collagen can also be observed using X-ray diffraction to observe the changes in axial packing of collagen molecules in fibers as dehydration occurs. Layered and/or crosslinked bioengineered constructs can be formed into a number of form factors, such as tubular constructs, based on well known techniques (see, for example, U.S. Patent No. 5,712,163 to Parenteau, PCT Publication No. WO 95/31473, PCT Publication No. WO 00/29553, and PCT Publication No. WO 2009/070720).

d. Combination Products

Other materials may be added to ECMs to further enhance bioactivity or function when administered *in vivo*.

Foe example, antimicrobial agents, drugs, growth factors, cytokines, genetic material and cultured cells can be incorporated in or on the bioengineered constructs, layers therein, and/or scaffolds.

Where the bioengineered constructs contact with blood in their use, as in the circulatory system, they can be rendered non-thrombogenic by applying heparin to the construct, to all surfaces of the construct or one side only in a flat-sheet construct or either luminally or abluminally for a tubular construct. Heparin can be applied to the construct,

by a variety of well-known techniques. For illustration, heparin can be applied to the construct in the following three ways. First, benzalkonium heparin (BA-Hep) isopropyl alcohol solution is applied to the prosthesis by vertically filling the lumen or dipping the prosthesis in the solution and then air-drying it. This procedure treats the collagen with an ionically bound BA-Hep complex. Second, EDC can be used to activate the heparin and then to covalently bond the heparin to the collagen fiber. Third, EDC can be used to activate the collagen, then covalently bond protamine to the collagen and then ionically bond heparin to the protamine.

Synthetic materials can be disposed upon at least one surface of the bioengineered constructs. The synthetic material can be in the form of a sheet, superimposed or staggered upon the bioengineered construct to form a synthetic layer on the bioengineered layer. One class of synthetic materials, preferably biologically compatible synthetic materials, comprises polymers. Such polymers include but are not limited to the following: poly(urethanes), poly(siloxanes) or silicones, poly(ethylene), poly(vinyl pyrrolidone), poly(2-hydroxy ethyl methacrylate), poly(N-vinyl pyrrolidone), poly(methyl methacrylate), poly(vinyl alcohol), poly(acrylic acid), polyacrylamide, poly(ethylene-co-vinyl acetate), poly(ethylene glycol), poly(methacrylic acid), polylactides (PLA), polyglycolides (PGA), poly(lactide-co-glycolid-es) (PLGA), polyanhydrides, and polyorthoesters or any other similar synthetic polymers that can be developed that are biologically compatible. The term "biologically compatible, synthetic polymers" also includes copolymers and blends, and any other combinations of the forgoing either together or with other polymers generally. The use of these polymers will depend on given applications and specifications required. For example, biologically compatible synthetic materials can also be biodegradable such that, when implanted into the body of a subject, biodegrade over time. When disposed on a bioengineered construct, the combination construct comprises a biodegradable layer and a bioremodelable layer. A more detailed discussion of these polymers and types of polymers is set forth in Brannon-Peppas, Lisa, "Polymers in Controlled Drug Delivery," Medical Plastics and Biomaterials, November 1997, which is incorporated by reference as if set forth fully herein.

An example of another synthetic material that can be used as a backing layer is silicone. A silicone layer in the form of a porous or microporous membrane or a non-porous film is applied and adhered to a matrix construct. When used in wound healing, the silicone layer can be used to handle and maneuver the matrix construct to a skin wound and

seal the wound periphery to enclose the matrix construct to treat the wound. The silicone also forms a moisture barrier to keep the wound from drying. Following successful formation of the healed wound tissue, typically at around 21 days, the silicone is peeled back carefully from the edges of the healed or healing wound with forceps.

Proteins can also be added to bioengineered constructs. Examples of useful extracellular matrix proteins include, but are not limited to, collagen, fibrin, elastin, laminin, and fibronectin, proteoglycans. Fibrinogen, when combined with thrombin, forms fibrin. Hyaluronan (also called hyaluronic acid or hyaluronate) is a non-sulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. It is one of the chief components of the extracellular matrix, contributes significantly to cell proliferation and migration and is used to reduce post-operative adhesions. There are multiple types of each of these proteins that are naturally-occurring, as well as types that can be or are synthetically manufactured or produced by genetic engineering. Collagen occurs in many forms and types. The term "protein" further includes, but is not limited to, fragments, analogs, conservative amino acid substitutions, and substitutions with non-naturally occurring amino acids with respect to each named protein. The term "residue" refers to an amino acid (D or L) or an amino acid mimetic that is incorporated into a protein by an amide bond. As such, the amino acid can be a naturally occurring amino acid or, unless otherwise limited, can encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (*i.e.*, amino acid mimetics). Moreover, an amide bond mimetic includes peptide backbone modifications well known to those skilled in the art. For example, peptides can be used to enhance cellular effects (*e.g.*, human dermal fibroblast infiltration into a silk scaffold and improve the ability to recruit host cells, such as epithelial cells). Such peptides could be RGD, Gofoger, laminin 1-10, and pronectin. More specifically, laminin 5 and laminin 10 function particularly well to increase epithelial cell infiltration/migration. Peptides can also be used to enhance endothelial cell migration. More particularly, peptides such as thrombin and fibrinogen can enhance endothelial cell migration, especially for indications benefiting from neovascularization.

Cell adhesion molecule can also be incorporated into or onto the polymer matrix to attach the scaffold composition to the local tissue site and prevent diffusion of the bioengineered construct. Such molecules are incorporated into the polymer matrix prior to polymerization of the matrix or after polymerization of the matrix. Examples of cell

adhesion molecules include but are not limited to peptides, proteins and polysaccharides such as fibronectin, laminin, collagen, thrombospondin 1, vitronectin, elastin, tenascin, aggrecan, agrin, bone sialoprotein, cartilage matrix protein, fibrinogen, fibrin, fibulin, mucins, entactin, osteopontin, plasminogen, restrictin, serglycin, SPARC/osteonectin, versican, von Willebrand Factor, polysaccharide heparin sulfate, connexins, collagen, RGD (Arg-Gly-Asp) and YIGSR (Tyr-Ile-Gly-Ser-Arg) peptides and cyclic peptides, glycosaminoglycans (GAGs), hyaluronic acid (HA), condroitin-6-sulfate, integrin ligands, selectins, cadherins and members of the immunoglobulin superfamily. Other examples include neural cell adhesion molecules (NCAMs), intercellular adhesion molecules (ICAMs), vascular cell adhesion molecule (VCAM-1), platelet-endothelial cell adhesion molecule (PECAM-1), L1, and CHL1.

ECM Proteins and peptides and role in cell function

Protein	Sequence	SEQ.ID No:	Role
Fibronectin	RGDS		Adhesion
	LDV		Adhesion
	REDV		Adhesion
Vitronectin	RGDV		Adhesion
Laminin A	LRGDN		Adhesion
	IKVAV		Neurite extension
Laminin B1	YIGSR		Adhesion of cells, via 67 kD laminin receptor
	PDSGR		Adhesion
Laminin B2	RNIAEIIKDA		Neurite extension
Collagen 1	RGDT		Adhesion of most cells
	DGEA		Adhesion of platelets and other cells
Thrombospondin	RGD		Adhesion of most cells
	VTXG		Adhesion of platelets

Additional examples of suitable cell adhesion molecules are shown below.

Amino acid sequences specific for proteoglycan binding from extracellular matrix proteins

SEQUENCE	SEQ.ID.NO.	PROTEIN
XBBXBX*		Consensus sequence
PRRARV		Fibronectin
YEKPGSPPREVVPRPRPGV		Fibronectin
RPSLAKKQRFRHRNRKGYRSQRGHSRGR		Vitronectin
rIQNLLKITNLRIKFVK		Laminin

Particularly preferred cell adhesion molecules are peptides or cyclic peptides

containing the amino acid sequence arginine-glycine-aspartic acid (RGD) which is known as a cell attachment ligand and found in various natural extracellular matrix molecules. A polymer matrix with such a modification provides cell adhesion properties to the scaffold, and sustains long-term survival of mammalian cell systems, as well as supporting cell growth.

Growth factors can also be introduced into the bioengineered constructs and/or onto the scaffold structures. Such substances include BMP, bone morphogenetic protein; ECM, extracellular matrix proteins or fragments thereof; EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PIGF, placental growth factor; TGF, transforming growth factor, VEGF, vascular endothelial growth factor, MCP1, and IL4. Cell-cell adhesion molecules (cadherins, integrins, ALCAM, NCAM, proteases, Notch ligands) are optionally added to the scaffold composition. Exemplary growth factors and ligands are provided in the tables below.

Growth factors used for angiogenesis

Growth factor	Abbreviation	Relevant activities
Vascular endothelial growth factor	VEGF	Migration, proliferation and survival of ECs
Basic fibroblast growth factor	bFGF-2	Migration, proliferation and survival of ECs and many other cell types
Platelet-derived growth factor	PDGF	Promotes the maturation of blood vessels by the recruitment of smooth muscle cells
Angiopoietin-1	Ang-1	Strengthens EC-smooth muscle cell interaction
Angiopoietin-2	Ang-2	Weakens EC-smooth muscle cell interaction
Placental growth factor	PIGF	Stimulates angiogenesis
Transforming growth factor	TGF	Stabilizes new blood vessels by promoting matrix deposition

Growth factors used for wound healing

Growth Factor	Abbreviation	Relevant activities
Platelet-derived growth factor	PDGF	Active in all stages of healing process
Epidermal growth factor	EGF	Mitogenic for keratinocytes
Transforming growth factor-β	TGF-β	Promotes keratinocyte migration, ECM synthesis and remodeling, and differentiation of epithelial cells
Fibroblast growth factor	FGF	General stimulant for wound healing

Growth Factors Used for Tissue- Engineering

Growth factor	Abbreviation	Moleular weight (kDa)	Relevant activities	Representative supplier of rH growth factor

Epidermal growth factor	EGF	6.2	Proliferation of epithelial, mesenchymal, and fibroblast cells	PeproTech Inc. (Rocky Hill, NJ, USA)
Platelet-derived growth factor	PDGF-AA PDGF-AB PDGF-BB	28.5 25.5 24.3	Proliferation and chemoattractant agent for smooth muscle cells; extracellular matrix synthesis and deposition	PeproTech Inc.
Transforming growth factor-α	TGF-α	5.5	Migration and proliferation of keratinocytes; extracellular matrix synthesis and deposition	PeproTech Inc.
Transforming growth factor-β	TGF-β	25.0	Proliferation and differentiation of bone forming cells; chemoattractant for fibroblasts	PeproTech Inc.
Bone morphogenetic protein	BMP-2 BMP-7	26.0 31.5	Differentiation and migration of bone forming cells	Cell Sciences Inc. (Norwood, MA, USA)
Basic fibroblast growth factor	bFGF/FGF-2	17.2	Proliferation of fibroblasts and initiation of angiogenesis	PeproTech Inc.
Vascular endothelial growth factor	$VEGF_{165}$	38.2	Migration, proliferation, and survival of endothelial cells	PeproTech Inc.

rH, recombinant human

Immobilized ligands used in tissue engineering

Immobilized ligand*	ECM molecule source	Application
RGD	Multiple ECM molecules, including fibronectin, vitronectin, laminin, collagen and thrombospondin	Enhance bone and cartilage tissue formation *in vitro* and *in vivo* Regulate neurite outgrowth *in vitro* and *in vivo* Promote myoblast adhesion, proliferation and differentiation Enhance endothelial cell adhesion and proliferation
IKVAV YIGSR RNIAEIIKDI	Laminin	Regulate neurite outgrowth *in vitro* and *in vivo*
Recombinant fibronectin fragment ($FNIII_{7-10}$)	Fibronectin	Promote formulation of focal contacts in pre-osteoblasts
Ac-GCRDGPQ-GIWGQDRCG	Common MMP substrates, (e.g. collagen, fibronectin, laminin)	Encourage cell-mediated proteolytic degradation, remodeling and bone regeneration (with RGD and BMP-2

		presentation) *in vivo*

*Sequences are given in single-letter amino acid code. MMP, matrix metalloproteinase.

In order to enhance blood vessel formation *in vivo*, devitalized bioengineered constructs can be soaked in proteins such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), hepatocyte growth factor/scatter factor (HGF/SF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and other kinds of pro-angiogenic factors. In one aspect, 50 micrograms of recombinant human PDGF-BB powder was reconstituted in 0.5 ml 4mM HCl, and then added with an additional 0.5 ml phosphate buffered saline (PBS). The resulting 1 mL solution was used to soak a devitalized bioengineered cosntruct prior to implantation in a full thickness wound in nude and normal mice. In addition, 50 micrograms of recombinant human basic fibroblast growth factor (bFGF) was reconstituted in 1 mL of PBS. Bioengineered constructs were soaked in the 1 mL bFGF solution for 5 minutes prior to implantation in a full thickness wound in nude and normal mice. In another embodiment, 50 micrograms of recombinant human PDGF-BB was reconstituted in 0.5 ml 4mM HCL and subsequently mixed with 0.5 mL PBS-reconstituted recombinant human bFGF. Bioengineered constructs were soaked in the resulting 1 mL solution for five minutes prior to implantation in a full thickness wound on nude and normal mice. In another embodiment, bioengineered constructs are produced as in Example 12. The conditioned culture medium from any of the multiple feeds over the course of the culture time can be collected. In particular, conditioned culture medium was collected after day 11 and concentrated (e.g. 100-fold). Devitalized bioengineered constructs of the invention were subsequently soaked in the concentrated conditioned media immediately prior to implantation.

Supplements can also be introduced into the chemically defined culture medium in order to selectively enhance desired extracellular matrix attributes and/or to achieve desired *in vivo* outcomes. Chemically defined culture medium comprises the following:

Component	Concentration (Volume for 1L)
DMEM	96.0% (960 mL in 1 L)
L-Glutamine	1060 mg/L
Hydrocortisone	0.4 mg/L
Selenious acid	6.78 μg/L
ITT (2.5 mg/mL Insulin +2.5 mg/mL	2 mL

Transferrin + 6.74 ng/mL Triiodothyronine)	
EOP ((3.103 g/L monoethalonamine +7.06 g/L o-Phosphorylethanolamine)	2 mL
EGF	10.0 µg/L
Mg Ascorbate	50 mg/L
L-Proline	213.6 mg/L
Glycine	101.4 mg/L
Long TGFα	20 ng/mL
Prostaglandin 2	0.038 µg/mL

In order to increase the amount of hyaluronic acid (HA) in the bioengineered construct and to enhance new blood vessel formation *in vivo,* the chemically defined culture medium can be supplemented with 2x long *TGF*α (40 ng/mL). Additionally, the chemically defined culture medium can further be supplemented with 25 ng/ml of PDGF at day 5, 25 ng/ml of bFGF at day 10, and 25 ng/ml of hepatocyte growth factor (HGF) at day 15. Alternatively, the chemically defined cultured media comprises supplementation with 2x long *TGF*α (40 ng/mL), 25 ng/ml of bFGF at day 5, 25 ng/ml of PDGF at day 10, and 25 ng/ml of bFGF at day 15. An additional alternative chemically defined media formulation is 2x long *TGF*α (40 ng/mL), 25 ng/ml of pDGF on day 5, 25 ng/ml bFGF on day 10, and 25 ng/ml of HGF on day 15.

Alternatively,, bioengineered constructs of the invention can be produced to comprise an elevated amount of sulfated glycosaminoglycans (sGAG) by supplementing the chemically defined culture medium to comprise 10x long *TGF*α (200 ng/mL). More particularly, when comparing bioengineered constructs produced by supplementing the chemically defined culture media with 10x long *TGF*α (200 ng/mL) and 1X *TGF*α (20 ng/mL), approximately 1100 ug sGAG/construct was observed in the bioengineered constructs produced by the medium supplemented with 10x long *TGF*α (200 ng/mL), as opposed to 600 ug sGAG/construct in bioengineered constructs produced in medium supplemented with 1X *TGF*α (20 ng/mL). It is to be appreciated that the changes in media supplementations disclosed herein can be used to treat the silk scaffold with or without HDFs seeded thereon without straying from the scope of the invention.

Bioengineered constructs can be treated with a surface modification to enhance the adhesiveness or tissue-attachment properties. The surface modification providing the

adhesive "means" can be included on the apical, basal, or both opposing surfaces which functions to increase the binding of a construct when intimately applied to a patient's tissues and organs *in vivo*. The adhesive-enhancing "means" can be one or more of any of the following: (a) the incorporation of a plurality of self-assembled microstructure and/or nanostructures molded onto and protruding from the bioengineered surface; (b) an added biocompatible and biodegradable adhesive material, such as a film, gel, hydrogel, liquid, or glue, bonded, coated or applied directly onto the bioengineered surface; or, (c) an electrospun sticky fiber matrix that is overlaid or spun onto the bioengineered surface.

The adhesive-enhancing means can be restricted to one outer surface (either basal or apical, depending on the preferred manufacturing design). This adhesive construct can be used for organ repairs, bulking, reinforcing or reconstruction. The adhesive construct is not meant to adhere to surrounding tissues adjacent to the wound but only to adhere directly to surface of the organ in need of healing. However, both basal and apical surfaces can contain an adhesive-enhancing means, either the same or different means on each surface, depending on the intended therapeutic use of the composition (*e.g.*, for purposely holding internal tissues or organs in tight proximity to one another, or alternatively, for adhering a patient's tissue tightly to the surface of an exogengous, implantable therapeutic device or sensor).

Certain manufacturing methods can be used to produce the various embodiments, whether they are made to contain the self-assembled micro- and/or nano-structures or are made to include the biocompatible and biodegradable adhesive materials. For example, the implant's shape can be a patch that is circular, oval, elliptical, triangular, or various sizes of rectangles and squares dependent on its intended therapeutic use (e.g. long, narrow rectangles for certain applications similar to a tape format where the composition has a length substantially larger than its width, for example for bone or other organ wraps, while other uses can require more square-like patches, for example, for hernia repair. The implant can be further trimmed by the surgeon, as necessary, to match the particular size and shape of the patient's defect. Furthermore, the tape or patch can include one or more drugs to discourage bacterial infection, such as colloidal silver or microbial toxins, and to discourage post-surgical bleeding, such as fibrinogen or thrombin. In a further embodiment, the construct could be mitotically inactivated by gamma irradiation, treatment with mitomycin-C, or any other means known in the art prior to shipment which would allow the donor cells to continue to secrete their biological healing factors but would

prevent their long term engraftment in the patient host. At least a portion of the adhesive article can have an adhesive strength of equal to or greater than approximately 0.05 Newton per square centimeter of projected area when measured according to ASTM standard D4501, D4541, or D6862-04.

Adhesive means include a plurality of self-assembled microstructures molded onto the basal surface of the bioengineered constructs produced with fibroblasts and/or mesenchymal progenitor cells unit that is formed by the cells and their secreted extracelluar matrix which mimic the modified pore surface of culture insert membranes of the bioreactor system. Essentially the plating system surface acts as as a micromold containing numerous engineered cavities or void structures where the cells would settle into these voids upon culturing and then secrete proteins, lipids, GAGs and other matrix factors to fill these voids, thereby creating the protusions or tissue "grippers" covering all or a portion of the basal surfaces of the bioengineered constructs which are formed in mirror image to the nanoscale topography of the plating surfaces upon removeal of the bioengineered constructs from the bioreactor. The microfabricated topography of the plating surfaces can be formed using a variety of techniques known in the art, including but not limited to, lithography, nanodrawing, microetching, and photolithography followed by etching or nanomolding. The protrusions can be formed in a variety of shapes and sizes including cones, spikes, cylinders, prisms, pyramids, polygonal, patterned grooves, suction cups, or the shapes that mimic the nanoscale setae and spatulae topography found on the footpads of geckos. The protrusions can include a second, a third or additional sets of protrusions extending from the main protrusions of the bioengineered construct basal or apical surface. The protrusions would be an inherent feature of the bioengineered constructs and can be uniform in their shape and size on a surface or can be arranged in combinations of shapes and sizes, depending on the intended use and level of adhesiveness required. The protrusions can be arranged in various patterns and in various densities on the surface. The density of protrusions, or the number of protrusions per unit area, ranges from approximately 10 protrusions/cm^2 to approximately $1x10^{10}$ protrusions/cm^2. The protrusions can be arranged in a pattern, or regularly, irregularly or randomly arranged, depending on the intended application of the tape or patch. In some embodiments the protrusions have an average height of less than approximately 1,000 micrometers. The protrusions can have an average height of from approximately 0.2 μm to approximately 150 μm. The protrusions can have an average tip width of from approximately 0.05 μm to approximately 150 μm. The

protrusions can have an average base width of from approximately 0.05 μm to approximately 150μm. The protrusions can have an average center-to-center pitch of from approximately 0.2μm to approximately 500 μm. The protrusions can have an average height to base width ratio of from approximately 0.1:1 to approximately 500:1. The protrusions can have an average base width to tip width ratio of from approximately 1000:1 to approximately 0.1:1. In some embodiments, the self-assembled protrusions can be capable of piercing the tissue of the patient upon application by the surgeon.

Alternatively, the adhesive-enhancing means is an adhesive material applied either to the surface of the bioreactor prior to initial plating of the cells, or alternatively, applied directly to the surface of the self-assembled bioengineered constructs after culturing has been completed but before final packaging (*i.e.*, post-liquid growth medium removal but prior to shipment of the units). Important features for adhesives useful in the invention include ones that are biodegradable, biocompatible, flexible, elastic, able to form strong bonds to tissue surfaces (even in moist or wet environments). The adhesive material should be capable of forming a chemical bond with the cell matrix construct's surface, such as a covalent bond or non-covalently through van der Waals, electrostatic, or hydrogen interactions. The adhesive material can be added to the surface of the construct either by spraying, rolling or dipping. A variety of adhesive materials known in the art can be used to form the adhesive surface including, but are not limited to cellulose, carboxymethyl cellulose, hydroxypropyl methyl cellulose or combinations thereof. Other materials for use in the adhesive surface can include but are not limited to poly(glycerol sebacate) (PGS), poly(glycerol sebacate acrylate) (PGSA), poly(lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL), polyglycolide (PGA), polylactic acid (PLA), poly-3-hydroxybutyrate (PHB), phosphoester polyamines, polyurethane, parylene-C, keratin, carbon nanotubes, poly(anhydride), polyvinylpyrrolidone, polypropylene glycol, hyaluronic acid, dextrans, collagen, chitin, chitosan, silk fibroin, glycosaminoglycans, fibrin, fibrinogen or the like.

The adhesive-enhancing means can also be made from nanofibers or microfibers having inherent adhesive properties that are electrospun directly onto the surface of the self-assembled constructs after culturing has been completed but before final packaging (i.e. post-liquid growth medium removal but prior to shipment of the units). The electrospun nanofibers or microfibers can be, but are not limited to, collagen, poly(lactic-co-glycolic

acid) (PLGA), polycaprolactone (PCL), polyglycolide (PGA), polylactic acid (PLA), and combinations thereof.

e. Meshed Bioengineered Constructs

Bioengineered constructs can also be meshed prior to grafting to a subject in need of wound care. When used in wound healing, meshing improves conformation to the wound bed and provides a means for draining wound exudate from beneath the graft. The term 'meshing' is defined as a mechanical method by which a tissue is perforated with slits to form a net-like arrangement. Meshed constructs can be expanded by stretching the skin so that the slits are opened and then applied to the wound bed. Expanded meshed constructs provide a wound area with maximal coverage. Alternatively, meshed constructs can be applied without expansion, simply as a sheet with an arrangement of unexpanded slits. The meshed construct can be applied alone or with the subject's own skin from another area of the body. Constructs can also have perforations or fenestrations and pores provided by other means. Fenestrations can be applied manually using a laser, punch, scalpel, needle or pin. Bioengineered constructs can also be provided with holes that communicate between both planes of the construct. Holes are perforations that are introduced in a regular or irregular pattern. One could also manually score or perforate a tissue with a scalpel or a needle.

f. Terminally Sterilized Bioengineered Constructs

Constructs can be terminally sterilized using means known in the art. A preferred method for sterilization is by contacting the constructs with sterile 0.1% peracetic acid (PA) treatment neutralized with a sufficient amount of 10 N sodium hydroxide (NaOH), according to U.S. Pat. No. 5,460,962, the disclosure of which is incorporated herein. Decontamination is performed in a container on a shaker platform, such as 1 L Nalge containers, for between 16-20 hours (*e.g.*, 18 hours). Constructs can then be rinsed by contacting them with three volumes of sterile water for 10 minutes each rinse. Constructs can be sterilized by gamma irradiation. Constructs can be packaged in containers made from material suitable for gamma irradiation and sealed using a vacuum sealer, which were in turn placed in hermetic bags for gamma irradiation between 15.0 and 40.0 kGy. Gamma irradiation significantly, but not detrimentally, decreases susceptibility to construct degradation, Young's modulus and shrink temperature. The mechanical properties after gamma irradiation are still sufficient for use in a range of applications and

gamma is a preferred means for sterilizing as it is widely used in the field of implantable medical devices.

V. TREATMENT METHODS AND MEDICAL USES

Bioengineered constructs, with or without cells, can be delivered to a subject, for example, to treat a damaged or diseased organ or tissue, to repair the organ or tissue and/or to restore its intended functionality. The bioengineered constructs of the invention have properties that, when implanted into a subject in a therapeutically effective amount, induce site-appropriate tissue repair and regeneration. A therapeutically effective amount of a construct can be provided to a subject in one or more administrations or applications. Due to the differentiation potential of mesenchymal progenitor cells, inclusion of these multipotential cell populations will improve the the rate and quality of healing of bone, cartilage, tendon, ligament, muscle, and skin). The bioengineered constructs would be angiogenic, anti-inflammatory, osteogenic, adipogenic or fibrogenic, or a combination thereof, when implanted adjacent to, or in contact with, the tissue or organ to be treated as appropriate for that site of implantation.

The bioengineered constructs of the invention have angiogenic properties, meaning that they induce the growth of new blood vessels, which is important for wound healing and formation of granulation tissue of cutaneous wounds and other surgical applications of the bioengineered constructs. Angiogenesis is detected by way of, for example, standard histology techniques (such as by way of αSMA staining) or other assays as disclosed herein.

The bioengineered constructs of the invention have anti-inflammatory properties when implanted, meaning that host inflammatory cell infiltration is minimized so that host cells rather will migrate into the implanted bioengineered construct for bioremodeling of the construct and repair of host tissue. Host cell migration from the host tissues into the implanted bioengineered construct will, as part of the regenerative healing response. Histological techniques can be used to determine the extent of inflammatory cell infiltration and host cell migration. The bioengineered constructs of the invention also have osteogenic properties, meaning that new bone formation will occur at a treatment site. Osteogenesis is measured by detection of new connective and ossifying tissue, higher cell activity detection and turnover of the newly formed tissues. Standard histology techniques and other techniques can be used to measure the cellular effect as well as bone density and bone surface area at the treatment site. The bioengineered constructs would be adipogenic,

forming new adipose (fat) tissue, when implanted at a treatment site. Fibrogenic properties of the bioengineered constructs may be realized when implanted at a treatment site. Bioengineered constructs of the invention can be used for a variety of human and non-human (i.e., veterinary) therapeutic applications.

The invention includes a medical uses and methods for treating subjects in need of wound healing using a bioengineered construct of the invention to treat surgical wounds; burn wounds; chronic wounds; diabetic lower extremity ulcers; venous ulcers; pressure ulcers (with or without negative pressure wound therapy); arterial ulcers; tunneling wounds such as those that tunnel away from a chronic wound cavity; sinuses (e.g., pilonidal, post-surgical dehiscences) and fistulae (e.g., anal, enterocutaneous, vesico-vaginal, oro-antral, broncho-pleural).

Other medical uses and methods for treating subjects in need of treatment include cardiac applications, applications to hard and soft tissues of the oral cavity (e.g., treatment of receded gingival tissue, guided bone regeneration to repair bone defects or deteriorated bone, guided tissue regeneration and repair of connective tissues of the oral cavity),

Additional medical uses and treatment methods for using the bioengineered constructs include cosmetic applications including dermal soft tissue fillers (e.g., contouring for cosmesis), breast reconstruction applications (e.g., augmentation, lift, and/or mastopexy) and neurological applications, such as a dura mater repair patch or a graft for peripheral nerve repair, a wrap for nerve bundles or tube for guided nerve regeneration.

Further uses of the bioengineered constructs include, but are not limited to, the application to suture lines or open wounds to improve the sealing and strength capabilites for certain surgical procedures where leakage of air or fluids would be detrimental to the subject's health and require additional corrective surgical procedures to prevent complications, such as infection, abscess formation, or internal bleeding (*e.g.*, gastric bypass; colostomies; stomach and large and small bowel resections; vascular grafts; vascular implants; coronary artery bypass grafts; abdominoplasty; abdominal surgeries (e.g. laparotomy); Caesarean-sections; tracheostomy sites; catheter implant sites; sealing of pericardium, pleura, and dural trauma); application as a prophylactic treatment to heal or prevent rupture of organs (e.g., vulnerable plaque stabilization; abdominal aortic/aneurysm ruptures; stomach or small intestine ulcer perforations; Crohn's disease; inflammatory bowel disease); "holes" that need filling for cell growth repair (e.g., urinary incontinence;

nose or septum repairs; anal fistulas; ostomies; muscle tears; cartilage tears; joint coating material; soft tissue and muscle wall hernia repairs;);

Still further uses of the bioengineered constructs include, but are not limited to bone grafts and repairs (e.g. compound fractures; osteotomies; artificial periosteal membrane; stump covering for limb and appendage amputations; foot and ankle fusions); cardiovascular tissue repair and regeneration (post-myocardial infarction; congestive heart failure); myocardial ischemia; stroke; peripheral arterial disease; neuropathies; coronary artery disease); nerve repair applications; liver regeneration applications (fibrosis; acute, subacute and chronic hepatisis; cirrhosis; fulminant hepatic failure; covering of the outer surface following lobe transplantation); kidney regeneration applications during acute renal failure; surgical wound closures; abdominal surgical adhesion prevention; cardiovascular, salivary duct, or bile-duct stent covering.

The bioengineered constructs can be applied or implanted to a treatment site by contacting it with damaged or diseased tissue, by filling a void in a tissue space or by placement where a subject's tissue does not or no longer exists. Application or implantation of the bioengineered constructs may be achieved through pressure touch directly to an organ's surface, via wrapping circumferentially around the organ, or affixed to the treatment site using surgical adhesvies, sutures or staples. The bioengineered constructs may alsp be delivered as a flat sheet, rolled, wadded, or injected to a treatment site. The bioengineered construct can be delivered intraoperably during open surgical procedures, percutaneously, or laparoscopically by passing the construct through a cannula to the defect. Regardless of the mode of delivery, the device functions to stimulate regenerative healing processes by locally delivering the repair building blocks and cell signalling compounds at relevant physiologic concentrations, including cells along with their complex array of secreted cytokines, ECM proteins, glycosaminoglycans, lipids, matrix reoganizing enzymes, and collagen materials which can be reorganized to meet the needs of the wounded organ or function to locally recruit the endogenous regenerative cells of the host. Alternatively, the bioengineered constructs would incorporate genetically modified cells that would function to deliver local cell-based gene therapy to certain organs of a subject in need thereof. The construct can also incorporate a drug to function as a drug delivery vehicle for small molecule therapeutics, biological therapeutics or pharmaceuticals for the internal, local, sustained, slow-release delivery of therapeutics to a subject in need thereof.

The following examples are provided to better explain the practice of the present invention and should not be interpreted in any way to limit the scope of the present invention. Those skilled in the art will recognize that various modifications can be made to the methods described herein while not departing from the spirit and scope of the present invention.

EXAMPLES

Example 1: Bioengineered Construct Produced by Mesenchymal Stem Cells (MSCs)

Generation of bioengineered constructs comprising mesenchymal stem cells grown under conditions to produce a layer of extracllular matrix which is synthesized and assembled by the mesenchymal stem cells is exemplified using human umbilical cord perivascular cells (HUCPVC). Specifically, skilled artisans have heretofore been unable to define preparatory conditions for allowing MSCs to synthesize and assemble extracellular matrix components to any appreciable thickness. Prior to seeding the HUCPVC, culture inserts were coated with about 5 ug/cm^2 of human plasma-derived fibronectin. The bioengineered constructs were produced by initially seeding 3 x 10^6 HUCPVC per 24 mm insert. Subsequent to seeding the cells upon a culture insert with a porous membrane in a insert, the cells were maintained in culture for 18 days, with replacement with fresh culture media at days 5, 8, 12, and 15, in the following chemically defined culture medium:

Component	Concentration (Volume for 1L)
DMEM	96.0% (960 mL in 1 L)
L-Glutamine	1060 mg/L
Hydrocortisone	0.4 mg/L
Selenious acid	6.78 µg/L
ITT (2.5 mg/mL Insulin +2.5 mg/mL Transferrin + 6.74 ng/mL Triiodothyronine)	2 mL
EOP (3.103 g/L monoethalonamine + 7.06 g/L o-Phosphorylethanolamine)	2 mL
EGF	10.0 µg/L
Mg Ascorbate	50 mg/L
L-Proline	213.6 mg/L
Glycine	101.4 mg/L

Long TGFα (Novozymes A/S)	200 ng/mL
Prostaglandin 2	0.038 μg/mL

The resulting bioengineered constructs generate extracellular matrices that are at least 30 microns thick. Time-course analysis of extracellular matrix formation was conducted to correlate MSC-derived bioengineered construct thickness with lengths of culture time. **Figures 1A** and **1B** demonstrate that the greatest increases in bioengineered construct thickness can be achieved by twelve days of culturing.

In order to further define the factors contributing to efficient extracellular matrix synthesis and assembly by mesenchymal stem cells, the role of TGF-alpha and prostaglandin 2 were assessed. **Figure 2** demonstrates the correlation between increasing bioengineered construct thickness as a function of increased TGF-alpha concentration in the culture media after culturing 3 x 10^6 HUCPVC per 24 mm insert for 18 days. **Figure 3** demonstrates the correlation between decreasing bioengineered construct thickness as a function of increased prostaglandin 2 concentration in the culture media after culturing 3 x 10^6 HUCPVC per 24 mm insert for 18 days. Accordingly, the amount of extracellular matrix synthesized and assembled by mesenchymal stem cells can be modulated based on culture media components and, in particular, appreciable thicknesses of the resulting bioengineered construct can be achieved. In addition, culture media supplementation can synergize with increased seeding densities (such as superconfluent densities containing 3 x 10^6 to 10 x 10^6 cells or more per 24 mm insert) to produce even thicker extracellular matrices in MSC-derived bioengineered constructs, including those derived from HUCPVC, bone marrow-derived MSCs, and pre-adipocytes (**Figure 4**). In a specific embodiment, superconfluent cell seeding was performed using 30 x 10^6 cells per 75 mm insert, which is equivalent to 9.6 x 10^6 cells per 24 mm insert.

Example 2: Biophysical Properties of Bioengineered Constructs Produced by Mesenchymal Stem Cells (MSCs)

In addition to generating appreciable amounts of synthesized and assembled extracellular matrix by mesenchymal stem cells to produce bioengineered construct having significant thicknesses, such bioengineered constructs have additional biophysical properties that distinguish them from extracellular matrices formed by other cell types.

MSC-derived bioengineered constructs seeded at superconfluency and cultured for 18 days according to the methods and culture media defined in Example 1.exhibited a significant difference in collagen arrangement and overall matrix morphology from

similarly cultured HDF-derived bioengineered constructs (except using 20 ng/mL TGF-alpha) . In particular, the extracellular matrix containes pore, is less dense, and contains aggregates of collagen bundles (**Figures 5A-5B**). Thus, MSC-derived bioengineered constructs have a porosity, which can be represented as the percentage area that is represented by pores in a histological section relative to the total area of the histological section. Such a porous extracellular matrix is desirable for many wound healing indications since it allows for greater migration and infiltration of host cells and angiogenesis-related molecules once grafted into a wound. However, such porous extracellular matrices should also maintain mechanical integrity to allow a physician to apply the bioengineered construct with minimal difficulty. Accordingly, mechanical testing of the MSC-derived and HDF-derived bioengineered constructs were performed to evaluate several mechanical properties. Specifically, Fmax (also known as Max load/Max force, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 N) is the maximum load that can be applied onto a material before it breaks. Ultimate tensile strength (also known as UTS, *e.g.*, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 N/cm^2) is the maximum pressure load sustained by a specimen prior to rupture. Elasticity modulus (also known as elongation, *e.g.*, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 Displacement/Initial length) is a measure of the stiffness of a material within the linear region whereby the material will return to a starting condition if the load is removed. **Figures 6A-6C** show that MSC-derived bioengineered constructs have similar mechanical integrity to HDF-derived bioengineered constructs despite having a more porous extracellular matrix, with HUCPVC-derived bioengineered constructs having the most similar mechanical integrity and thickness profile.

A bioengineered construct having a porous extracellular matrix with strong mechanical properties would be further useful for treating wounds by allowing the diffusion growth factors at the delivery site that promote wound healing. In order to characterize differences in extracellular matrix components, adhesion components, and/or growth factors present between the MSC-derived bioengineered constructs and those generated using other cell types, quantitative PCR (qPCR) assays were performed using cDNA isolated from MSC-derived seeded at superconfluency and cultured for 18 days (according to the methods and culture media defined in Example 1) or human dermal fibroblast (HDF)-derived bioengineered constructs seeded at superconfluency and cultured for 18 days (according to the methods and culture media defined in Example 1, except that the culture media was supplemented with 20 ng/mL long TGF-α). Real-time PCR primers from the

Human ECM and Adhesion Molecules Array (SuperArray PAHS-013A) and the Human Growth Factor Array (SuperArray PAHS-041A) according to the manufacturer's protocol. **Figure 7** shows a summary of differences in growth factors between MSC-derived and HDF-derived bioengineered constructs. For example, the increased collagen expression in HUCPVC-derived bioengineered constructs is consistent with the collagen bundling characteristics observed in Figure 5. Increased expression of CXCL6, a chemoattractive molecule; KDR, an indicator of VEGF-induced proliferation, migration, tubular morphogenesis, and endothelial sprouting; and laminin alpha 5 (LAMA5), an indicator of embryonic cell organization, was also observed in HUCPVC-derived bioengineered constructs. These results demonstrate that, in addition to the appreciable thicknesses of extracellular matrix achieved using MSC-derived bioengineered constructs, such constructs also exhibit upregulation of genes useful in treating a wound environment, such as promoting healing rates and angiogenesis (**Figure 7**).

In addition, protein-based assays for detecting IL-6, IL-8, and VEGF levels using the cytometric bead array system (CBA) from Becton Dickinson were performed using MSC-derived bioengineered constructs seeded at superconfluency and cultured for 18 days (according to the methods and culture media defined in Example 1) or human dermal fibroblast (HDF)-derived bioengineered constructs seeded at superconfluency and cultured for 18 days (according to the methods and culture media defined in Example 1, except that the culture media was supplemented with 20 ng/mL Long TGFα), according to the manufacturer's protocol. **Figures 8A-8C** show a time-course comparison of IL-6, IL-8, and VEGF levels within the conditioned media generated by the MSC-derived and HDF-derived bioengineered constructs. IL-6 expression in MSC-derived bioengineered constructs peaked early during the culture time-course and was over 9 times that of the HDF-derived bioengineered constructs at day 5 of culturing HUCPVC-derived bioengineered constructs (**Figure 8A**). Besides its role in the immune response, IL-6 is also secreted by osteoblasts to promote osteoclast formation. IL-8 expression was also significantly overexpressed in MSC-derived bioengineered constructs relative to HDF-derived bioengineered constructs over the entire length of culture (**Figure 8B**). Besides its role in the immune response, IL-8 is also secreted by epithelial cells as a potent angiogenic factor since via binding to such receptors as CXCR1 and CXCR2. Similarly, VEGF is another potent angiogenic factor and is significantly overexpressed in MSC-derived bioengineered constructs relative to HDF-derived bioengineered constructs during the early

phases of culture (**Figure 8C**). It is believed that the drop in detectable VEGF levels in the culture media are due to the high levels of KDR expression by HUCPVCs and other MSCs, which is the receptor for VEGF and sequesters the molecules within the bioengineered construct so as to preclude detection in the media. In addition, CSF-3 and vitronectin are upregulated in HUCPVC-derived bioengineered constructs relative to HDF-derived bioengineered constructs. An ELISA assay was further performed on conditioned media samples of culturing HDF-derived and MSC-derived bioengineered constructs according to the methods of Example 1 (*i.e.*, 10x TGF-alpha for both conditions) to quanify the amount hyaluronan (HA) production after 5 and 18 days. **Figure 8D** shows that whereas HA levels in culture media of HDF-derived bioengineered constructs decreased from 4,664 ng/mL on day 5 to 4,085 ng.mL on day 18, HA levels in culture media of HUCPVC-derived bioengineered constructs increased from 4,333 ng/Ml on day 5 to 5,615 ng/mL on day 18. In addition, MSC-derived bioengineered constructs exhibited 38-fold more vitronectin, 21-fold more CSF-3, 15-fold more NCAM1, and 4-fold more CXCL1 relative to HDF-derived bioengineered constructs.

Finally, MSC-derived bioengineered constructs seeded at superconfluency and cultured for 18 days according to the methods and culture media defined in Example 1, yielded a conditioned medium having components that increase the ability for cells to migrate relative to HDF-derived bioengineered constructs cultured under identical conditions, except that the culture media was supplemented with 20 ng/mL Long TGFα (**Figure 9**).

Example 3: Multilineage Potential Properties of Bioengineered Constructs Produced by Mesenchymal Stem Cells (MSCs)

Assays were performed to determine the multilineage potential properties of cells isolated from bioengineered constructs produced by MSCs, as well as from MSCs within the native bioengineered construct environment. MSC-derived bioengineered constructs were seeded at superconfluency and cultured for 18 days according to the methods and culture media defined in Example 1. At day 18, the bioengineered constructs were either digested with collagenase to determine cell yields and cell digests for multilineage potential assays or directly cultured in induction media. Non-induced MSC control groups of cells and bioengineered constructs were maintained for each of the induced cell and bioengineered construct groups, wherein alpha MEM media supplemented with 10% fetal bovine serum (FBS) was used in the place of induction media. Media changes occurred

every 2-3 days. In addition, HDF-derived control groups of cells and bioengineered constructs were maintained for each of the induced cell and bioengineered construct groups.

For the osteogenic induction assay, bioengineered constructs were directly cultured in osteogenic induction media and cells resulting from collagenase digestion were seeded at 20,000 cells/cm^2 in 12-well plates for osteogenic induction. The defined culture media shown in Example 1 was replaced at day 18 of culture with the following osteogenic induction media: Complete DMEM base media supplemented with 10^{-3} M dexamethasone (DEX), 1M β-glycerophosphate (BGP), and 50 mg/mL ascorbic acid (AA). Osteogenic induction culturing occurred for days prior to analysis of gene expression of Runx2 (a transcription factor expressed in the later stages of osteoblastic differentiation), ALP, and osteoclacin (OC) using RNA isolated from the bioengineered constructs or cultured cells. An 8-fold increase in the expression of ALP was observed in the induced MSC-derived bioengineered construct relative to non-induced MSC-derived bioengineered constructs (**Figure 10A**). In addition, an 11-fold increase in the expression of Runx2 was observed in isolated MSC-derived bioengineered construct cells that were induced in osteogenic induction media relative to such cells that were not induced in osteogenic induction media (**Figure 10B**). Thus, MSCs within an intact bioengineered construct or isolated from such constructs can be induced toward an osteognic lineage based on environmental signaling cues.

For the adipogenic induction assay, bioengineered constructs were directly cultured in adipogenic induction media and cells resulting from collagenase digestion were seeded at 20,000 cells/cm^2 in 12-well plates for adipogenic induction. The defined culture media shown in Example 1 was replaced at day 18 of culture with the following adipogenic induction media: Complete DMEM base media supplemented with 10^{-3} M dexamethasone (DEX), 10 mg/mL insulin, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). Osteogenic induction culturing occurred for days prior to analysis of neutral triglycerides and lipids from the bioengineered constructs or cultured cells using standard Oil Red-O staining. Only isolated MSC-derived bioengineered construct cells that were induced in adipogenic induction media were observed to have a significant number of positively stained cells relative to such cells that were not induced in adipogenic induction media (**Figure 10C**). Thus, MSCs within an intact bioengineered construct or isolated from such constructs can be induced toward an adipogenic lineage based on environmental signaling cues.

Thus, MSCs within and isolated from an intact bioengineered construct can be

induced toward several cell lineages based on environmental signaling cues while a sub-population is maintained with stem-like potential.

Example 4: *In vivo* Vascularization Properties of Bioengineered Constructs Produced by Mesenchymal Stem Cells (MSCs)

The purpose of this study was to graft bioengineered constructs produced by the methods of Example 1 onto nude mice, and analyze their response *in vivo* when implanted subcutaneously. More particularly, alpha-Smooth Muscle Actin (αSMA) staining was used to qualitatively and quantitatively analyze vascularization within the construct in the mice. Units were grafted in a subcutaneous implantation model in female Swiss nude mice at 8 weeks old.

After 1 week following subcutaneous implantation of the various bioengineered constructs, 5 animals from each group listed in the following table were sacrificed:

Groups	Time-points	Total # units/readouts	# of units per groups
A:100% MSC-derived bioengineered construct	- 1 week - 4 weeks	3 x 24 mm for Histology 3 x 24 mm for qPCR (stem cell panel) 6 x 24 mm for CFU-F and FC (post-digesting) 10 x 14 mm punch for grafting (but round up to 3 plates/group)	5 plates/group (30 units)
B:50% admixed bioengineered construct			
C:10% admixed bioengineered construct			
D: (control) 100% HDF-derived bioengineered construct			

The implant area was removed and processed for histological examination. In particular, histological sections from n=2 animals from each group were stained with αSMA. **Figures 11A-11D** show representative sections taken of αSMA stained sections from 100% HUCPVC-derived bioengineered constructs, 50% HUCPVC-50% HDF-derived bioengineered constructs, 10% HUCPVC-90% HDF-derived bioengineered constructs, and 100% HDF-derived bioengineered constructs, respectively. All bioengineered constructs were produced as described in Example 1, with the exception of 100% HDF-derived constructs were cultured with 20 ng/mL TGF-alpha. The bioengineered constructs in **Figure 11A** appear to have a more pronounced number of αSMA positive staining within the implant area as compared to the constructs of **Figures 11B-11D**. The αSMA staining is specifically associated around newly formed vessels, which is clearly seen in **Figure 11A** at 40x magnification. Quantification of αSMA revealed that the 100% HUCPVC produced

bioengineered constructs had greater numbers of vessels within the implant area related to the other groups (**Figure 11D**). While not wishing to be bound by theory, the HUCPVC can secrete cytokines/growth factors, such as those describe above in Examples 2 and 3 that act in a paracrine fashion to recruit mouse endothelial cells, which then subsequently form new vessels. In addition, the matrix and its associated organization that is generated by the HUCPVC can provide a more suitable provisional matrix for cell recruitment and infiltration into the implant area, leading to the higher vessel formation seen at 1 week relative to other groups. Additionally, standard angiogenesis assays can be performed to further confirm the increased ability of HUCPVC-derived bioengineered constructs to promote angiogenesis, such as assaying for the ability of constructs to form and/or maintain tubule from endothelial cells (*e.g.*, an angiogenesis tube formation assay from Millipore) and gene expression analyses of angiogenesis biomarkers (*e.g.*, angiogenesis ELISA assays from Q-Plex and, angiogenesis proteome profiler array assays from R&D Systems).

Example 5: Controlling Contraction of Bioengineered Constructs

Bioengineered constructs were produced by seeding human neonatal foreskin fibroblasts onto 75 mm membrane inserts with plasma-treated (COOH) PES membranes that comprise 5 micron pores. The initial cell-seeding density was 30 million cells per membrane insert. Cells were suspended in chemically defined culture media (containing no undefined non-human components) with 20 ml of suspension seeded directly onto the insert, and 110 ml of media in the culture reservoir to allow for bilateral feeding of the cells. The medium contained: a base 3:1 mixture of DMEM, 2mM L-Glutamine (Invitrogen Inc.)4 mM GlutaMAX (Gibco BRL, grand Island, NY) and additives: 5 ng/ml human recombinant epidermal growth factor (Upstate Biotechnology, Lake Placid, NY), 1 x 10^{-4} M ethanolamine (Fluka, Ronkonkoma, NY cat. #02400 ACS grade), 1 x 10^{-4} M o-phosphoryl-ethanolamine (Sigma, St. Louis, MO), 5 ug/ml transferrin (Sigma, St. Louis, MO), 20 pM triiodothyronine (Sigma, St. Louis, MO), and 6.78 ng/ml selenium (Sigma Aldrich Fine Chemicals Company, Milwaukee, WI), 50 ng/ml L-ascorbic acid (WAKO Chemicals USA, Inc.), 0.2 ug/ml L-proline (Sigma, St. Louis, MO), 0.1 ug/ml glycine (Sigma, St. Louis, MO), 20 ng/ml TGF-alpha (*i.e.*, 1x TGF-alpha) and 10 nM PGE_2. The cells were cultured in this manner for 18 days prior to harvesting the bioengineered constructs. In some embodiments, 2x TGF-alpha or more can be preferable. Several bioengineered constructs were immediately formalin-fixed for histology analysis so as to prevent natural contraction (**Figure 12**), whereas the remaining bioengineered constructs

were control contracted, as described further below.

Specifically, sterile forceps were used to detach the bioengineered constructs from the Transwell membrane such that they were left floating in the culture dish. In order to produce a porous bioengineered construct while still retaining strong mechanical properties, the bioengineered constructs were contracted in a controlled manner by returning the floating constructs to an incubator and allowing the bioengineered constructs to naturally contract for two hours. After two hours, the media was removed, rinsed in RODI water, and formalin-fixed for histology analysis (**Figure 13**). Bioengineered constructs having undergone controlled contraction (**Figure 12**) show an approximately 2-fold increase in average bioengineered construct thickness (*e.g.*, 400-800 μm average thicknesses versus 200-300 μm average thicknesses) relative to those not having undergone controlled contraction (**Figure 13**).

In another embodiment, after the two hours of floating incubation, the bioengineered constructs were subsequently soaked in 1 mM EDC solution at 4°C overnight, though the construct could alternatively be soaked in 0.2 mM EDC, 0.5 mM EDC, 5mM EDC, or 10 mM EDC in the culture dishes without straying from the scope of the present invention. After EDC cross-linking, the construct was rinsed with reverse osmosis deionized (RODI) water three times, drained and laid flat. After rinsing with RODI water, the bioengineered constructs were cooled down from room temperature (~ 20°C) at a rate of 0.5°C per minute for 2 hours until a final freezing temperature of -40°C was reached. After the bioengineered construct reached a temperature of -40°C, the bioengineered construct was annealed at -40°C for at least 2 hours. All bioengineered constructs were then subjected to a vacuum environment of lower than 200 mTorr in a lyophilizing apparatus and treated for twenty four hours at 0°C. It is to be appreciated that the freezing cycle can be performed in an appropriately-enabled lyophilizing apparatus or in any freezer, such as a control-rate freezer. It is to be further appreciated that the bioengineered constructs can be subjected to a vacuum environment between 0 mTorr and 350 mTorr without straying from the scope of the invention. In an alternative embodiment, theconstruct was allowed to air dry for 8 hours after EDC cross-linking without undergoing lyophilization (*i.e.*, freeze drying).

In another embodiment, after the two hours of floating incubation, the media was removed, and the bioengineered constructs were rinsed in MES buffer until the constructs no longer had a pink color. The constructs were then soaked in reverse osmosis deionized

(RODI) water for about one hour before being drained and laid flat. After rinsing with RODI water, the bioengineered constructs were cooled down from room temperature (~ 20°C) at a rate of 0.5°C per minute for 2 hours until a final freezing temperature of -40°C was reached. After the bioengineered construct reached a temperature of -40°C, the bioengineered construct was annealed at -40°C for 2 hours. All constructs were then subjected to a vacuum environment of lower than 200 mTorr in a lyophilizing apparatus for twenty four hours at 0°C. The bioengineered constructs were subsequently placed in a vacuum oven for 24 hours at 100°C to form dehydrothermal crosslinks (DHT) in the bioengineered constructs. In some embodiments, lyophilization can be preferred in the absence of crosslinking steps.

Example 6: Bioengineered Constructs Have *In Vivo* Osteogenic and Barrier Function

Bioengineered constructs as those produced using the methods of Example 5 (*i.e.*, EDC crosslinked, DHT crosslinked, and uncrosslinked bioengineered constructs, collectively referred to in this Example as the "test constructs") in addition to a negative control (no construct) and a positive control (a 25x25 mm standard bioabsorbable barrier membrane of Bioguide, which comprises a porcine type I and III collagen membrane from Osteohealth, One Luitpold Drive, P.O. Box 9001, Shirley, NY 11967) were implanted into each of the four quandrants of the jaw of Gottingen minipigs (maxillary right, maxillary left, mandibular right and mandibular left).

Specifically, Four male adult minipigs were co-housed in a separate room throughout the study at a temperature of 22 +/- 2°C. Each pig was anesthetized for 8 hours, during which time all bony defects were prepared and treated. The surgical procedure for applying each construct took approximately 2 hours. The second and fourth premolar teeth were extracted after 1) elevation of a full thickness gingival flap, 2) separation of the roots using a multiblade bur, and 3) incision of the periodontal ligament with an Orban scalpel. Before extractions, the buccal plate of the alveolar bone surrounding the teeth were penetrated with a round bur at various points and were cut using a carbide fissure bur by connecting the round bur holes. The buccal plate was surgically removed using bone chisels and bone scissors to create bone defects (1.2 cm^2 each). All constructss were 25 x 25 mm sections and were placed in randomly selected 4 maxillary and 4 mandibular sites so as to extend the mesial, distal and apical borders of the defect by 2-3 mm. Ligatures were used to tie the construct borders to the surrounding host gingival soft tissue. All surgical procedures were performed in aseptic conditions and using general anesthesia and

endotracheal intubation provided by LASC veterinary services.

After 4, 8, and 12 weeks, the designated animals were sacrificed and the test/control sites were retrieved together with the adjacent bone in block sections and fixed in a 10% formalin solution. Half of the block sections at each group were decalcified using a decalcification agent. After decalcification and dehydration, the blocks were immersed in paraffin, and subsequently 5 micrometer sections were cut and stained with hematoxylin-eosin for light microscopy and identification of the cellular composition of inflammatory infiltratem as well as for histopathologic and histomorphologic examination. Sections were also stained with masson's trichrome to detect new collagen deposition and new bone formation. The other half of the block sections were fixed in 4% formalin solution after scraping away the overlying soft tissue, dehydrated in ascending grades of alcohol, and embedded in methylmethacrylate for future staining with toluidine blue for evaluation of new bone and collagen deposits. Alveolar bone structure and newly formed tissue compositions were examined by quantitative microcomputed tomography (MicroCT) following defect treatment. MicroCT Scans were performed using a Scanco microCT 80 system (Scanco Medical, Bassersdorf, Switzerland) located at the Boston University Orthopaedic and Development Biomechanics laboratory at the Department of Mechanical Engineering. Immediately before scanning, the jaws of the 4 minipigs were removed from storage and allowed to calibrate to room temperature.

The test sites treated with test constructs showed higher cell activity and turnover of newly formed tissues, i.e., connective and osteoid tissues). At 8 weeks, healthy connective tissue and highly organized newly formed osteoid tissue filled the defect areas and the contours of buccal bone were almost fully reformed. At 12 weeks, the test sites treated with test constructs showed almost complete healing with well-connected new bone formation with the old bone while some sections showed continued healing with some osteoclasts at the bone surface indicating bone turnover.

Example 7: Controlling Pore Size of Bioengineered Constructs

The average pore size within the extracellular matrix of the bioengineered constructs of the invention can be engineered to form dense or porous extracellular matrix. Combined with a type and/or degree of crosslinking, defined average pore sizes can be chosen and controlled to yield constructs that have different rates of *in vivo* persistence and/or cell infiltration, ranging from "rapidly bioremodelable" to "moderately bioremodelable" to "prolonged bioremodelable" bioengineered constructs for tailored

applicability to therapeutic uses (**Figure 14A**). HDF-derived bioengineered constructs produced according to the methods of Example 5, were analyzed after 18 days in culture to determine the pore size and distribution characteristics. **Figure 13** demonstrates that such bioengineered constructs that have not been lyophilized have essentially no pores. However, bioengineered constructs were further subjected to controlled contraction, lyophilization, and either not crosslinked, crosslinked with EDC, or crosslinked using DHT methods according the methods of Example 5. The magic wand tool of the Scandium® image analysis program (Olympus) was used to statistically analyze pores lengths and areas on representative histological sections. Since pores are not precise circles, pore diameter was back calculated assuming the measured area of a given pore was derived from a circle. Two histology images per group were used to generate the measurements. **Figure 14B** shows that ramping to a final freezing temperature of -40°C, at a rate of 0.5°C per minute, resulted in average pore sizes of between 15 and 20 μm. In addition, **Figure 14C** further demonstrates that average pore size is determined by the final freezing temperature irrespective of crosslinking status. By contrast, **Figure 14D** shows that ramping the bioengineered constructs to a final freezing temperature of -10°C, which is a warmer freezing temperature than -40°C, at a rate of 0.5°C per minute, resulted in average pore sizes of at least 50 μm (*e.g.*, ranging between 30 μm and 100 μm). **Figure 14E** further demonstrate that the average pore size is independent of controlled contraction. Specifically, HDF-derived bioengineered constructs produced according to the methods of Example 5, and which were simply air dried after controlled contraction, produced a dense matrix with very small pores, if any). By contrast, bioengineered constructs that were processed as those shown in **Figure 14B** produced average pore sizes of between 15 and 20 μm. Similarly, the average pore size of MSC-derived bioengineered constructs generated according to the methods of Example 1 (**Figure 14F**) can be increased upon controlled contraction, rinsing, freezing from room temperature to -20°C, and lyophilization (**Figure 14G**).

Example 8: Controlling Bioengineered Construct Thickness and ECM Composition

HDFs were seeded at superconfluency (i.e., 30 x 10^6 cells per 75 mm insert) and cultured for 18 days according to the methods of Example 1, except that 20 ng/mL TGF-alpha was used. Heparin was also supplemented in the media at 5 μg/mL. To test the effect of basic fibroblast growth factor (bFGF; Peprotech Inc.) on the resulting bioengineered constructs, bFGF was supplemented and maintained in the culture media either upon initial

seeding or after 5 days in culture. **Figure 15A** shows that supplementing the chemically defined culture media with 20 ng/mL bFGF significantly reduced bioengineered construct thicknesses that were more easily tearable when handled with forceps relative to controls. Heparin supplementation had no effect on bioengineered construct thicknesses. Bioengineered constructs produced using 2 ng/mL bFGF had thicknesses similar to untreated controls.

The thinner bFGF-supplemented bioengineered constructs indicated that the extracellular matrix contained less matrix protein, less glycosaminoglycans, or both. **Figure 15B** shows the results of bFGF dose response analysis in which collagen accumulation decreased as bFGF supplementation increased. Since collagen populations form sequentially during extracellular matrix production (*i.e.*, reversibly crosslinked acid-soluble collagen, then pepsin-soluble collagen that is irreversibly crosslinked and must be isolated by cutting off crosslinks with pepsin, and then SDS soluble collagen, which is higly crosslinked and is neither acid-norpepsin-soluble), each of these populations collagen populations were extracted from control and bFGF-supplemented bioengineered constructs using standard techniques. The total collagen accumulation in the bFGF-supplemented bioengineered constructs is lower relative to controls and there is an especially significant deficiency in the accumulation of pepsin soluble collagen (**Figure 15B**). Heparin alone did not affect collagen accumulation.

Acid- and pepsin-soluble collagen amounts were independently assayed and quantified using a Sircol collagen assay on the bioengineered constructs analyzed in Figure 15B. Since SDS-soluble collagen is not triple helical, the Sircol assay does not detect this class of collagen. **Figure 15C** shows relative levels of both acid- and pepsin-soluble collagen (black) relative to total collagen and other collagen (grey). The combined amount of acid- and pepsin-soluble collagen in bioengineered constructs supplemented with 20 ng/mL or 100 ng/mL bFGF was 20% and 35%, respectively, of control amounts.

Differential scanning calorimetry (DSC) was subsequently performed to determine the total number of protein crosslinks in the bFGF-supplemented bioengineered constructs relative to controls. The peak area in bioengineered constructs supplemented with bFGF at either seeding or after 5 days in culture was decreased or zero relative to controls supplemented with heparin alone, indicating fewer crosslinks in the bFGF-supplemented bioengineered constructs.

In addition to changes in collagen amounts, sulfated glycosaminoglycan (sGAG),

which are responsible for binding growth factors and help regulate ECM hydration, as well as hyaluronic acid (HA), accumulated to lower levels in bFGF-supplemented bioengineered constructs relative to controls (**Figures 15D and 15E**). Histological staining assays independently confirmed that the bFGF-supplemented bioengineered constructs were less dense, contained less sGAG (Alcian blue staining), and contained fewer elastic fibers (van Gieson staining).

The alterations in extracellular matrix composition caused the bFGF-supplemented bioengineered constructs to turn to powder when dehydrated indicating that such constructs could easily be micronized by grinding. Bioengineered constructs produced using 20 ng/mL cracked when lyophilized in a temperature-controlled freeze dryer cracked during lyophilization, but the fragments remained as pliable as control units. However, the fragments were also less thick and significantly more porous than the control units. Immediately prior to lyophilization, the bFGF-supplemented bioengineered constructs were placed in a -80°C freezer for 2 hours. It is to be appreciated that the bFGF-supplemented bioengineered constructs could be kept in a freezer ranging in temperature from -10°C to -80°C anywhere from 1 hour to 3 days without straying from the scope of the invention. Alternatively, the bFGF-supplemented bioengineered constructs can be taken out of culture and placed directly into the lyophilizer. All bFGF-supplemented bioengineered constructs were then subjected to a vacuum environment of lower than 200 mTorr in a lyophilizing apparatus and treated for twenty four hours at 0°C. It is to be appreciated that the bioengineered constructs can be subjected to a vacuum environment between 0 mTorr and 350 mTorr without straying from the scope of the invention. In another embodiment, the bFGF-supplemented bioengineered constructs can be air-dried overnight at room temperature instead of treated in a lyophilizer.

The air-dried powder or lyophilized bFGF-supplemented bioengineered constructs, as well as controls, were micronized by grinding either using a mortar and pestle at room temperature or a tissue mill in which the constructs were kept frozen in liquid nitrogen. Similar amounts of ground constructs were rehydrated in phosphate buffered saline (PBS) in a microcentrifuge tube for 10 minutes prior to observation of fluid consistency. Rehydrated bFGF-supplemented constructs were significantly less viscous and floated more freely than control samples. This translated into an enhanced ability of the rehydrated bFGF-supplemented constructs to pass through a syringe needle (*i.e.*, they could pass through 23 gauge and 27 gauge, but not 30 gauge needles, whereas controls could not pass

through any such gauged syringe needles). Since scanning electron microscopy at 1000x magnification have determined that the particles in ground bFGF-supplemented constructs relative to controls are similar in size, it is believed that the viscosity of control particles impedes their passage through syringe needles. It is further believed that a finer or more consistent particle size can be achieved using finer tissue mills such that the rehydrated bFGF-supplemented constructs can pass through even finger gauged syringe needles.

Example 9: Porous Silk Scaffolds For Use With Bioengineered Constructs

Porous silk-based scaffolds were fabricated from degummed silk fiber of a Bombyx mori silkworm cocoon. The silk fibers were dissolved in 9 M LiBr solution at 6-10 wt % concentration for 6-10 hours while stirring under room conditions. The solution was dialyzed against water using a cellulose dialysis membrane for 3 days, changing the water every 10 hours. The fibroin aqueous solution was concentrated by standing the solution in a cellulose dialysis membrane. Insoluble portions were removed by centrifugation at 20,000 rpm for 30 minutes. The final concentration of the silk solution was about 7.5-8%.

The silk stock solution was then used to prepare a silk working solution with a concentration of 6% to 8%. The working solution was used to make a porous silk scaffold. The working solution was initially mixed with 1-6% ethanol solution with various volume ratios to make the final silk concentrations ranging from 3% to 5% and ethanol final concentrations ranging from 0.5% to 2%. The mixture was subsequently poured into a petri dish and placed into a -20°C freezer for at least 10 hours. After the 10 hours has passed, the silk solution was placed at room temperature and allowed to thaw, resulting in a porous silk scaffold. The thawed silk scaffolds were subsequently rinsed in RODI water for 3 days to remove solvent residue. After rinsing, a top thin layer can be removed from the surface of the scaffolds. Silk scaffolds can be sterilized by autoclaving the final scaffold, or using autoclaved silk solution mixed with sterile filtered ethanol solution, or using sterile filtered silk solution mixed with sterile filtered ethanol solution.

In order to enhance blood vessel formation *in vivo*, porous silk scaffolds can be soaked in proteins such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), hepatocyte growth factor/scatter factor (HGF/SF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and other kinds of pro-angiogenic factors. In one aspect, 50 micrograms of recombinant human PDGF-BB powder was reconstituted in 0.5 ml 4mM HCl, and then added with an additional 0.5 ml phosphate buffered saline (PBS). The resulting 1 mL solution was used to soak a 6x6 mm silk scaffold prior to

implantation in a full thickness wound in nude and normal mice. In addition, 50 micrograms of recombinant human basic fibroblast growth factor (bFGF) was reconstituted in 1 mL of PBS. 6x6 mm porous silk scaffolds were soaked in the 1 mL bFGF solution for 5 minutes prior to implantation in a full thickness wound in nude and normal mice. Also, 50 micrograms of recombinant human PDGF-BB was reconstituted in 0.5 ml 4mM HCL and subsequently mixed with 0.5 mL PBS-reconstituted recombinant human bFGF. Porous silk scaffolds were soaked in the resulting 1 mL solution for five minutes prior to implantation in a full thickness wound on nude and normal mice. In addition, silk scaffolds can be cultured with cells in chemically defined culture media comprising supplementation with 25 ng/ml of PDGF at day 5, 25 ng/ml of bFGF at day 10, and 25 ng/ml of hepatocyte growth factor (HGF) at day 15. Alternatively, the chemically defined cultured media comprises supplementation with 25 ng/ml of bFGF at day 5, 25 ng/ml of PDGF at day 10, and 25 ng/ml of bFGF at day 15 or 25 ng/ml of pDGF on day 5, 25 ng/ml bFGF on day 10, and 25 ng/ml of HGF on day 15. Also, the conditioned culture medium applied to bioengineered constructs on day 11 of Example 10 can be concentrated (e.g. 100-fold) and silk scaffolds can be soaked in the conditioned media.

In one embodiment, human dermal fibroblasts were seeded onto the porous silk scaffold. Specifically, human dermal fibroblasts were initially seeded at about 30 x 10^6 and cultured in chemically defined medium for 11 days. Alternatively, it is to be appreciated that HDFs can be seeded atop the silk scaffold at an initial seeding density of about 5 x 10^6. The chemically defined medium comprised: a base 3:1 mixture of DMEM, Hams F-12 medium (Quality Biologics, Gaithersburg, MD), 4 mM GlutaMAX (Gibco BRL, grand Island, NY) and additives: 5 ng/ml human recombinant epidermal growth factor (Upstate Biotechnology, Lake Placid, NY), 1 x 10^{-4} M ethanolamine (Fluka, Ronkonkoma, NY cat. #02400 ACS grade), 1 x 10^{-4} M o-phosphoryl-ethanolamine (Sigma, St. Louis, MO), 5 ug/ml transferrin (Sigma, St. Louis, MO), 13.5 pg/mL triiodothyronine (Sigma, St. Louis, MO), and 6.78 ng/ml selenium (Sigma Aldrich Fine Chemicals Company, Milwaukee, WI), 50 ng/ml L-ascorbic acid (WAKO Chemicals USA, Inc.), 0.2 ug/ml L-proline (Sigma, St. Louis, MO), 0.1 ug/ml glycine (Sigma, St. Louis, MO), 20 ng/ml TGF-alpha and 10 nM PGE_2. As can be seen in **Figure 16**, the human dermal fibroblasts were able to migrate through the silk scaffolds and are uniformly disposed throughout the silk sheet.

Several modifications can be made to engineer desired characteristics into the resulting bioengineered constructs cultured on porous silk scaffolds.

In another embodiment, the silk scaffolds having cultured HDFs were devitalized by rinsing the silk scaffolds comprising cultured HDFs with WFI water. For indications that require an enhanced angiogenic response, silk scaffolds having an average pore diameter of 50-100 microns, seeded with HDFs, and resulting in WFI water-devitalized bioengineered constructs have been shown to be an effective treatment. More specifically, **Figure 17(d)** shows stained human umbilical vein endothelial cells atop the silk scaffolds with devitalized fibroblasts *in vitro*. The stained endothelial cells form aligned tubules atop the silk scaffolds, an indication that the silk scaffolds with devitalized fibroblasts allow for effective endothelial cell attachment and persistence.

In another embodiment, the bioengineered constructs containing porous silk scaffolds and devitalized HDFs were subsequently crosslinked with EDC in order to make a bioengineered tissue construct with enhanced in vivo persistence (e.g., in a burn wound bed).

Silk scaffolds can also be impregnated with useful molecules. Silk scaffolds were submerged in pre-conditioned, chemically defined culture medium previously collected (post-culture) from endogenously produced bioengineered tissue constructs to enhance the silk scaffolds. More specifically, about 30 million human dermal fibroblasts were cultured atop a 0.4 micrometer porous membrane and cultured in chemically defined medium for 11 days. The chemically defined medium comprises: a base 3:1 mixture of DMEM, Hams F-12 medium (Quality Biologics, Gaithersburg, MD), 4 mM GlutaMAX (Gibco BRL, grand Island, NY) and additives: 5 ng/ml human recombinant epidermal growth factor (Upstate Biotechnology, Lake Placid, NY), 1 x 10^{-4} M ethanolamine (Fluka, Ronkonkoma, NY cat. #02400 ACS grade), 1 x 10^{-4} M o-phosphoryl-ethanolamine (Sigma, St. Louis, MO), 5 ug/ml transferrin (Sigma, St. Louis, MO), 13.5 pM triiodothyronine (Sigma, St. Louis, MO), and 6.78 ng/ml selenium (Sigma Aldrich Fine Chemicals Company, Milwaukee, WI), 50 ng/ml L-ascorbic acid (WAKO Chemicals USA, Inc.), 0.2 ug/ml L-proline (Sigma, St. Louis, MO), 0.1 ug/ml glycine (Sigma, St. Louis, MO), 20 ng/ml TGF-alpha and 10 nM PGE_2. After the 11 days in culture, the conditioned medium was collected, and the silk scaffolds were soaked in the conditioned medium for 12 hours.

A silicone backing can also be applied to one or both sides of a silk scaffold to act as a barrier to preventing infection while allowing transport of gaseous molecules, such as oxygen. For example, silk scaffolds with devitalized human dermal fibroblasts were treated with a silicone coating. The silicone coating was optimized by varying the monomer

concentration to crosslinker concentration ratio during polymerization of silicone. The ratio of the monomer to the crosslinker can range from about 5 to 1 to about 20 to 1. For a wet silk sponge, the optimal monomer to crosslinker ratio is about 5 to 1. In addition, the produced bioengineered construct can itself subsequently be coated with a silicone backing.

Enhancement of epithelial cell migration can be achieved by bathing the silk scaffolds in a solution of phosphate buffered saline and laminin 5 for about 1 hour. Depending on the porosity profile of the silk scaffold, the scaffold can be submerged in the laminin 5 solution for up to 4 hours. The silk scaffold with conjugated laminin 5 can be employed *in vivo* to enhance epithelial cell migration.

Example 10: Layered Constructs of HDFs and MSCs

Human neonatal foreskin fibroblasts (originated at Organogenesis, Inc. Canton, MA) were seeded at 5 x 10^5 cells/162 cm^2 tissue culture treated flask (Costar Corp., Cambridge, MA, cat # 3150) and grown in culture medium. The growth medium consisted of: Dulbecco's Modified Eagle's medium (DMEM) (high glucose formulation, without L-glutamine, BioWhittaker, Walkersville, MD) supplemented with 10% newborn calf serum (NBCS) (HyClone Laboratories, Inc., Logan, Utah) and 4 mM L-glutamine (BioWhittaker, Walkersville, MD). The cells were maintained in an incubator at 37 ± 1 °C with an atmosphere of 10 ± 1% CO_2. The medium was replaced with freshly prepared medium every two to three days. After 8 days in culture, the cells had grown to confluence, that is, the cells had formed a packed monolayer along the bottom of the tissue culture flask, and the medium was aspirated from the culture flask. To rinse the monolayer, sterile-filtered phosphate buffered saline was added to the bottom of each culture flask and then aspirated from the flasks. Cells were released from the flask by adding 5 mL trypsin-versene glutamine (BioWhittaker, Walkersville, MD) to each flask and gently rocking to ensure complete coverage of the monolayer. Cultures were returned to the incubator. As soon as the cells were released 5 ml of SBTI (Soybean Trypsin Inhibitor) was added to each flask and mixed with the suspension to stop the action of the trypsin-versene. The cell suspension was removed from the flasks and evenly divided between sterile, conical centrifuge tubes. Cells were collected by centrifugation at approximately 800-1000 x g for 5 minutes.

Cells were resuspended using fresh medium to a concentration of 3.0 x 10^6 cells/ml, and seeded onto 0.4 micron pore size, 24 mm diameter tissue culture treated inserts (TRANSWELL®, Corning Costar) in a six-well tray at a density of 1.0 x 10^6 cells/insert. It

is to be appreciated that if a 75 mm insert is to be used, a cell seeding density of 10 x 10^6 cells should be employed. If a 24 mm diameter insert is used, about 1 x 10^6 cells/24 mm insert should be employed. It is to be appreciated that the amount of HUCPVC were added to the suspension as a percentage of the amount of fibroblasts. For example, to make a layered 24 mm construct containing 50% HUCPVC, 5 x 10^5 HUCPVC were seeded atop the 1.0 x 10^6 human neonatal foreskin fibroblasts previously seeded atop the porous membrane. Both fibroblasts and HUCPVC were submerged in 3 ml of matrix production media, which comprises:

Component	Concentration
DMEM	96.0%
L-Glutamine	1060 mg/L
Hydrocortisone	0.4 mg/L
Selenious acid	6.78 µg/L
Ethanolamine	0.1 mM
o-Phosphorylethanolamine	14.0 Mg/L
EGF	10.0 µg/L
Mg Ascorbate	50 mg/L
L-Proline	213.6 mg/L
Glycine	101.4 mg/L
Long TGFα	10.0 µg/L

The cells were maintained in an incubator at 37 ± 1°C with an atmosphere of 10 ± 1% CO_2 and cultured in the matrix production medium for 11 days with media changes made periodically, every 3-4 days.

The formalin fixed samples were embedded in paraffin and 5 micrometer sections were punched and then stained with hematoxylin-eosin (H&E) according to procedures known in the art. Using H&E stained slides, thickness measurements were made to ten randomly picked microscopic fields utilizing a 10X eyepiece loaded with a 10 mm/100 micrometer reticle.

Example 11: Production of Bioengineered Constructs By Admixing HDFs and MSCs

A construct having a fibroblasts and HUCPVC-produced extracellular matrix layer were formed in a fully chemically defined culture media system. 1 x 10^5 human neonatal

dermal fibroblasts are seeded in a mixed cell population with 9 x 10^5 mesenchymal progenitor cells on a 24 mm culture insert. It is to be appreciated that the initial seeding density of the fibroblasts can range from about 1 x 10^5 to about 9 x 10^5 and the initial seeding density of the mesenchymal progenitor cells can also range from about 1 x 10^5 to about 9 x 10^5 within the scope of the invention. HUCPVC were obtained at passage 2, and expanded to passage 7 prior to being initially seeded upon the culture insert. It is to be appreciated that the HUCPVC can be used at any other passage number so long as the multipotentiality of the cells is preserved.

The chemically defined matrix production medium contained:

Component	Concentration
DMEM	96.0%
L-Glutamine	1060 mg/L
Hydrocortisone	0.4 mg/L
Selenious acid	6.78 µg/L
Ethanolamine	0.1 mM
o-Phosphorylethanolamine	14.0 Mg/L
EGF	10.0 µg/L
Mg Ascorbate	50 mg/L
L-Proline	213.6 mg/L
Glycine	101.4 mg/L
Long TGFα	10.0 µg/L

Fibroblasts and mesenchymal progenitor cells were cultured in the matrix production medium for 11 days with media changes made periodically, every 3-4 days, resulting in an endogenously produced extracellular matrix.

Example 12: Production of An Epidermal Layer On Bioengineered Constructs

Human epidermal progenitor cells (HEP's; keratinocytes) are seeded atop the bioengineered constructs described in any one of Examples 1-8. HEP's are seeded after the bioengineered constructs have been in culture for about 11 days. A seeding density of about 3.5 x 10^5 - 1.2 x 10^6 cells/construct is preferred, however other initial seeding densities are also contemplated in accordance with the invention. At day 11, the skin-constructs with the HEP's are treated with a medium containing approximately:

Component	Concentration
DMEM:HAM's F-12 3:1	96.10%
L-Glutamine	1060 mg/L
Hydrocortisone	0.4 mg/L
Insulin	5.0mg/L
Transferrin	5.0 mg/L
Triiodothyronine	13.5 ng/L
Ethanolamine	0.1 mM
o-Phosphorylethanolamine	14.0 Mg/L
Selenious acid	6.78 µg/L
Adenine	24.4 mg/L
Mg Ascorbate	50.0 mg/L
Progesterone	0.63 µg/L
EGF	10.0 µg/L
Long TGFα	10.0 µg/L
Lipid Concentrate	Arachidonic Acid 0.004 mg/L Cholesterol 0.220 mg/L DL-α-Tocopherol-Acetate 0.140 mg/L Linoleic Acid 0.020 mg/L Linolenic Acid 0.020 mg/L Myristic Acid 0.020 mg/L Oleic Acid 0.020 mg/L Palmitoleic Acid 0.020 mg/L Palmitic Acid 0.020 mg/L Pluronic® F-68 200.0 mg/L Stearic Acid 0.020 mg/L Tween® 80 4.4 mg/L

At day 13, differentiation of the HEP's are induced by using a differentiation medium containing the following:

Component	Concentration
DMEM:HAM's F-12 3:1	96.3%

L-Glutamine	1060 mg/L
Hydrocortisone	0.40 mg/L
Insulin	5.0 mg/L
Transferrin	5.0 mg/L
Triiodothyronine	13.5 ng/L
Selenious acid	0.00678 mg/L
Ethanolamine	0.1 mM
o-Phosphorylethanolamine	14.0 Mg/L
Adenine	24.4 mg/L
Mg Ascorbate	50.0 mg/L
Progesterone	0.63 μg/L
CaCl2	265 mg/L
Lipid Concentrate	Arachidonic Acid 0.004 mg/L Cholesterol 0.220 mg/L DL-α-Tocopherol-Acetate 0.140 mg/L Linoleic Acid 0.020 mg/L Linolenic Acid 0.020 mg/L Myristic Acid 0.020 mg/L Oleic Acid 0.020 mg/L Palmitoleic Acid 0.020 mg/L Palmitic Acid 0.020 mg/L Pluronic® F-68 200.0 mg/L Stearic Acid 0.020 mg/L Tween® 80 4.4 mg/L

At day 15, the medium formulation is changed to induce cornification of the developing epithelial layer in a medium containing approximately:

Component	Concentration
DMEM	48.0%
HAM's F-12	48.0%
L-Glutamine	658 mg/L
Hydrocortisone	0.4 mg/L

Insulin	5.0 mg/L
Transferrin	5.0 mg/L
Triiodothyronine	13.5 ng/L
Ethanolamine	0.1 mM
o-Phosphorylethanolamine	14.0 Mg/L
Selenius acid	6.78 μg/L
Adenine	24.4 mg/L
Mg Ascorbate	50.0 mg/L
Long TGFα	10.0 μg/L
MEM Non-Essential Amino Acid Solution	L-Alanine: 1.78 mg/L L-Asparagine: 2.64 mg/L L-Aspartic Acid: 2.66 mg/L L-Glutamic Acid: 2.94 mg/L Glycine: 1.5 mg/L L-Proline: 2.3 mg/L L-Serine: 2.1 mg/L
MEM Vitamin Solution	NaCl: 17 mg/L D-Ca Pantothenate: 0.2 mg/L Choline Chloride: 0.2 mg/L Folic Acid: 0.2 mg/L i-Inositol: 0.4 mg/L Nicotinamide: 0.2 mg/L Pyridoxal HCl: 0.2 mg/L Riboflavin: 0.020 mg/L Thiamine HCl: 0.2 mg/L
Lipid Concentrate	Arachidonic Acid: 0.004 mg/L Cholesterol: 0.220 mg/L DL-α-Tocopherol-: 0.140 mg/L

Acetate	
Linoleic Acid	0.020 mg/L
Linolenic Acid	0.020 mg/L
Myristic Acid	0.020 mg/L
Oleic Acid	0.020 mg/L
Palmitoleic Acid	0.020 mg/L
Palmitic Acid	0.020 mg/L
Pluronic® F-68	200.0 mg/L
Stearic Acid	0.020 mg/L
Tween® 80	4.4 mg/L

Cornfication medium is changed every 2-3 days. Bioengineered constructs are matured and maintained during days 22 through 35 and are fed a maintenance medium with changes every 2-3 days with fresh maintenance medium containing:

Component	Concentration
DMEM	48.0%
HAM's F-12	48.0%
L-Glutamine	658 mg/L
Hydrocortisone	0.4 mg/L
Insulin	5.0 mg/L
Transferrin	5.0 mg/L
Triiodothyronine	13.5 ng/L
Ethanolamine	0.1 mM
O-phosphorylethanolamine	14.0 Mg/L
Selenius acid	6.78 μg/L
Adenine	24.4 mg/L
Long TGFα	10.0 μg/L

MEM Non-Essential Amino Acid Solution	L-Alanine	1.78 mg/L
	L-Asparagine	2.64 mg/L
	L-Aspartic Acid	2.66 mg/L
	L-Glutamic Acid	2.94 mg/L
	Glycine	1.5 mg/L
	L-Proline	2.3 mg/L
	L-Serine	2.1 mg/L
MEM Vitamin Solution	NaCl	17 mg/L
	D-Ca Pantothenate	0.2 mg/L
	Choline Chloride	0.2 mg/L
	Folic Acid	0.2 mg/L
	i-Inositol	0.4 mg/L
	Nicotinamide	0.2 mg/L
	Pyridoxal HCl	0.2 mg/L
	Riboflavin	0.020 mg/L
	Thiamine HCl	0.2 mg/L
Lipid Concentrate	Arachidonic Acid	0.004 mg/L
	Cholesterol	0.220 mg/L
	DL-α-Tocopherol-Acetate	0.140 mg/L
	Linoleic Acid	0.020 mg/L
	Linolenic Acid	0.020 mg/L
	Myristic Acid	0.020 mg/L
	Oleic Acid	0.020 mg/L
	Palmitoleic Acid	0.020 mg/L
	Palmitic Acid	0.020 mg/L
	Pluronic® F-68	200.0 mg/L
	Stearic Acid	0.020 mg/L

	Tween® 80	4.4 mg/L	

When the bioengineered constructs are fully formed, the cultured bioengineered constructs exhibit a mixed bioengineered layer of endogenously produced extracellular matrix proteins, fibroblasts and/or mesenchymal progenitor cells with a differentiated epithelial layer disposed atop the bioengineered construct.

Example 13: Etching of Bioengineered Tissue Construct to Improve Cell Infiltration

Bioengineered tissue constructs can be modified to enhance cell attachment and cell infiltration within the deep network of pores on endogenously produced tissue constructs. Such endogenously produced constructs can be produced by initially seeding about 30 million human dermal fibroblasts atop a 0.4 micrometer porous membrane and cultured in chemically defined medium for 11 days. The chemically defined medium comprises: a base 3:1 mixture of DMEM, Hams F-12 medium (Quality Biologics, Gaithersburg, MD), 4 mM GlutaMAX (Gibco BRL, grand Island, NY) and additives: 5 ng/ml human recombinant epidermal growth factor (Upstate Biotechnology, Lake Placid, NY), 1 x 10^{-4} M ethanolamine (Fluka, Ronkonkoma, NY cat. #02400 ACS grade), 1 x 10^{-4} M o-phosphoryl-ethanolamine (Sigma, St. Louis, MO), 5 ug/ml transferrin (Sigma, St. Louis, MO), 20 pM triiodothyronine (Sigma, St. Louis, MO), and 6.78 ng/ml selenium (Sigma Aldrich Fine Chemicals Company, Milwaukee, WI), 50 ng/ml L-ascorbic acid (WAKO Chemicals USA, Inc.), 0.2 ug/ml L-proline (Sigma, St. Louis, MO), 0.1 ug/ml glycine (Sigma, St. Louis, MO), 20 ng/ml TGF-alpha and 10 nM PGE_2. After the 11 days of culture, the surface of the bioengineered tissue constructs can be etched to remove cell debris. This can be done by applying a solution of 1% acetic acid in order to remove a thin layer of collagen from the top surface of the bioengineered construct. Etching can allow for improved cell infiltration, which can be advantageous in a burn indication.

WE CLAIM:

1. A bioengineered construct comprising mesenchymal stem cells grown under conditions to produce a layer of extracellular matrix, which is synthesized and assembled by the mesenchymal stem cells.

2. The bioengineered construct of claim 1, wherein the mesenchymal stem cells are derived from bone marrow, umbilical cord, placenta, amnion, muscle, adipose, bone, tendon or cartilage.

3. The bioengineered construct of claim 1 or 2, wherein the mesenchymal stem cells are umbilical cord mesenchymal stem cells.

4. The bioengineered construct of claim 3, wherein the umbilical cord mesenchymal stem cells are isolated from umbilical cord blood, umbilical vein subendothelium, or Wharton's jelly.

5. The bioengineered construct of claim 3, wherein the umbilical cord mesenchymal stem cells are human umbilical cord perivascular cells (HUCPVC).

6. The bioengineered construct of any one of claims 1-5, wherein the mesenchymal stem cells are human mesenchymal stem cells.

7. The bioengineered construct of any one of claims 1-6, wherein the mesenchymal stem cells are transfected cells, recombinant cells, or genetically engineered cells.

8. The bioengineered construct of any one of claims 1-7, further comprising cells that are not mesenchymal stem cells, optionally wherein the non-mesenchymal stem cells are fibroblasts.

9. The bioengineered construct of claim 8, wherein the fibroblasts are derived from tissue selected from the group consisting of neonate male foreskin, dermis, tendon, lung, urethra, umbilical cord, corneal stroma, oral mucosa, and intestine.

10. The bioengineered construct of claim 8 or 9, wherein the fibroblasts are human fibroblasts.

11. The bioengineered construct of any one of claims 1-10, wherein the mesenchymal

stem cells and fibroblasts are admixed.

12. The bioengineered construct of any one of claims 1-10, wherein the mesenchymal stem cells and fibroblasts are present in at least two separate layers.

13. The bioengineered construct of any one of claims 1-12, wherein the extracellular matrix is at least 60 microns thick.

14. The bioengineered construct of any one of claim 1-13, wherein the bioengineered construct has pores in the range between 10 microns and 150 microns in diameter, optionally wherein the pores are in the range between 50 microns and 100 microns or between 80 microns and 100 microns.

15. The bioengineered construct of any one of claim 1-14, wherein the bioengineered construct has an average Fmax of at least 0.4 Newtons.

16. The bioengineered construct of any one of claims 1-15, wherein the bioengineered construct has an ultimate tensile strength (UTS) of at least 0.4 Megapascals.

17. The bioengineered construct of any one of claims 1-16, wherein the bioengineered construct has a plastic deformation tolerance of at least 0.4 times the initial length.

18. The bioengineered construct of any one of claims 1-17, wherein the cells of the bioengineered construct are devitalized.

19. The bioengineered construct of any one of claims 1-18, wherein the bioengineered construct is decellularized.

20. The bioengineered construct of any one of claims 1-19, wherein the bioengineered construct is dehydrated.

21. The bioengineered construct of any one of claims 1-20, wherein the extracellular matrix is crosslinked with a crosslinking agent.

22. The bioengineered construct of claim 21, wherein the crosslinking agent is selected from the group consisting of: carbodiimides, genipin, transglutaminase, ribose and other sugars, nordihydroguaiaretic acid (NDGA), oxidative agents and ultraviolet (UV) light.

23. The bioengineered construct of any one of claims 1-22, wherein bioengineered construct further comprises one or more of Hyaluronan, CSF-3, Vitronectin, heparin, NCAM1, CXCL1, IL-6, IL-8, VEGFA, VEGFC, PDGFβ, PECAM1, CDH5, ANGPT1, MMP2, TIMP1, and TIMP3.

24. The bioengineered construct of any one of claims 1-23, wherein the bioengineered construct further comprises an antimicrobial agent, a pharmaceutical drug, a growth factor, a cytokine, a peptide, or a protein.

25. The bioengineered construct of any one of claims 1-24, wherein the bioengineered construct is contracted to at least 50% decrease in surface area by releasing the bioengineered construct from the culture substrate.

26. The bioengineered construct of any one of claims 1-25, further comprising a porous silk fibroin scaffold upon which the mesenchymal stem cells grown under conditions to produce a layer of extracellular matrix are cultured.

27. The bioengineered construct of claim 26, wherein the porous silk fibroin scaffold has pores in the range between 10 microns and 150 microns in diameter.

28. The bioengineered construct of claim 26 or 27, wherein the porous silk fibroin scaffold has two sides and is coated with silicone on at least one side.

29. The bioengineered construct of any one of claims 26-28, wherein the porous silk fibroin scaffold further comprises an antimicrobial agent, a pharmaceutical drug, a growth factor, a cytokine, a peptide, or a protein.

30. The bioengineered construct of any one of claims 1-29, wherein the bioengineered construct further comprises an adhesive-enhancing means.

31. The bioengineered construct of any one of claims 1-30, wherein the bioengineered construct is terminally sterilized.

32. A multilayered bioengineered construct, wherein at least two bioengineered constructs of any one of claims 1-31 are bonded together.

33. The multilayered bioengineered construct of claim 32, wherein the bonded bioengineered constructs are crosslinked with a crosslinking agent.

34. A method for producing a bioengineered construct having an extracellular matrix with increased average pore size comprising:

a) seeding cells capable of synthesizing extracellular matrix components within a culture vessel;

b) culturing the cells to synthesize, secrete and organize extracellular matrix components;

c) lyophilizing at least the resulting extracellular matrix components, wherein lyophilization comprises freezing the extracellular matrix components to a final freezing temperature and subsequently drying the extracellular matrix components, thereby producing a bioengineered extracellular matrix construct having an extracellular matrix with increased average pore size.

35. The method of claim 34, wherein the average pore size of the porous extracellular matrix is increased by increasing the final freezing temperature.

36. The method of claim 34 or 35, wherein the bioengineered construct average pore size increases from at least 10 microns to at least 50 microns as the final freezing temperature increases from about -40°C to about -10°C.

37. The method of any one of claims 34-36, wherein the extracellular matrix producing cells are derived from neonate male foreskin, dermis, tendon, lung, umbilical cords, cartilage, urethra, corneal stroma, oral mucosa, intestine, bone marrow, placenta, amnion, muscle, adipose, or bone.

38. The method of any one of claims 34-37, wherein the extracellular matrix producing cells are human dermal fibroblasts or human umbilical cord perivascular cells.

39. The method of any one of claims 34-38, wherein the extracellular matrix producing cells are transfected cells, recombinant cells, or genetically engineered cells.

40. The method of any one of claims 34-38, wherein the bioengineered construct comprises at least one cell type in addition to the extracellular matrix producing cell type.

41. The method of claim 40, wherein the at least one additional cell type is selected from the group consisting of fibroblasts, stromal cells, and mesenchymal stem cells.

42. The method of claim 40 or 41, wherein the extracellular matrix producing cells and at least one additional cell type are admixed.

43. The method of any one of claims 40-42, wherein the extracellular matrix producing cells and at least one additional cell type are present in at least two separate layers.

44. The method of any one of claims 34-43, wherein the cells of each cell type are seeded at a combined density of between 1 x 10^5 cells/cm^2 to 6.6 x 10^5 cells/cm^2.

45. The method of any one of claims 34-43, wherein the cells of each cell type are seeded at a combined density of greater than 100% confluence.

46. The method of any one of claims 34-45, wherein the extracellular matrix is at least 60 microns thick before lyophilizing.

47. The method of any one of claims 34-46, wherein the cells of the bioengineered construct are devitalized or decellularlized before lyophilizing.

48. The method of any one of claims 34-47, wherein a final freezing temperature of about -40°C is reached to produce average pore sizes of at least 10 microns.

49. The method of any one of claims 34-48, wherein a final freezing temperature of about -10°C is reached to produce average pore sizes of at least 30 microns.

50. The method of any one of claims 34-49, wherein the extracellular matrix of the bioengineered construct is crosslinked with a crosslinking agent.

51. The method of claim 51, wherein the crosslinking agent is selected from the group consisting of: carbodiimides, genipin, transglutaminase, ribose and other sugars, nordihydroguaiaretic acid (NDGA), oxidative agents, dehydrothermal (DHT), and ultraviolet (UV) light.

52. The method of any one of claims 34-51, wherein the bioengineered construct is contracted to at least 50% decrease in surface area by releasing the bioengineered construct

from the culture substrate before lyophilizing.

53. The method of any one of claims 34-52, further comprising culturing the extracellular matrix producing cells upon a porous silk fibroin scaffold.

54. The method of claim 53, wherein the porous silk fibroin scaffold has pores in the range between 10 microns and 150 microns in diameter.

55. The method of claim 53 or 54, wherein the porous silk fibroin scaffold has two sides and is coated with silicone on at least one side.

56. The method of any one of claims 53-55, wherein the porous silk fibroin scaffold further comprises an antimicrobial agent, a pharmaceutical drug, a growth factor, a cytokine, a peptide, or a protein.

57. The method of any one of claims 34-56, wherein at least two bioengineered constructs are bonded together.

58. The method of claim 57, wherein the bonding occurs through an adhesive-enhancing means or by crosslinking with a crosslinking agent.

59. The method of any one of claims 34-58, wherein the bioengineered construct is terminally sterilized after lyophilizing.

60. The method of any one of claims 34-59, wherein the cells are cultured in chemically defined media.

61. The method of claim 60, wherein the chemically defined media is free of undefined animal organ or tissue extracts.

62. The method of claim 60 or 61, wherein the chemically defined media comprises TGF-alpha.

63. The method of any one of claims 34-62, wherein the cells are cultured on a porous membrane.

64. The method of claim 63, wherein the porous membrane comprises pores that are

less than 6 microns in size.

65. The method of any one of claims 34-64, wherein the rate of reaching the final freezing temperature is decreased to increase the uniformity of average pore sizes.

66. The method of claim 65, wherein the rate of reaching the final freezing temperature is between 0.1°C and 0.5°C per minute.

67. A bioengineered construct comprising:

extracellular matrix-producing cells;

endogenous extracellular matrix produced by the extracellular matrix-producing cells;

wherein the extracellular matrix-producing cells are devitalized.

68. The bioengineered construct of claim 67, wherein the bioengineered construct has pores in the range between 10 microns and 150 microns in diameter, optionally wherein the pores are in the range between 50 microns and 100 microns or between 80 microns and 100 microns.

69. The bioengineered construct of any one of claims 67-68, wherein the bioengineered construct is formed by cells cultured in chemically defined media.

70. The bioengineered construct of claim 69, wherein the chemically defined media comprises TGF-alpha.

71. The bioengineered construct of any one of claims 69-70, wherein the chemically defined media further comprises basic fibroblast growth factor (bFGF).

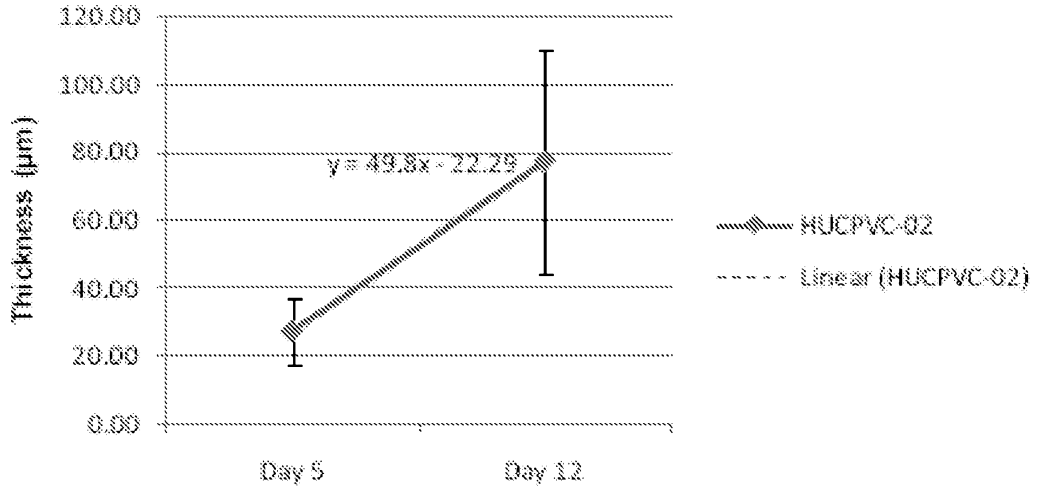
72. The bioengineered construct of any one of claims 67-71, wherein the extracellular matrix of the bioengineered construct is crosslinked with a crosslinking agent.

73. The bioengineered construct of claim 72, wherein the crosslinking agent is selected from the group consisting of: carbodiimides, genipin, transglutaminase, ribose and other sugars, nordihydroguaiaretic acid (NDGA), oxidative agents, dehydrothermal (DHT), and ultraviolet (UV) light.

74. The bioengineered construct of any one of claims 67-73, wherein the bioengineered construct is in powdered form.

Figure 1

A


120.00
100.00
80.00
60.00
40.00
20.00
0.00
Thickness (µm)
y = 49.8x - 22.29
Day 5
Day 12
HUCPVC-02
Linear (HUCPVC-02)

B

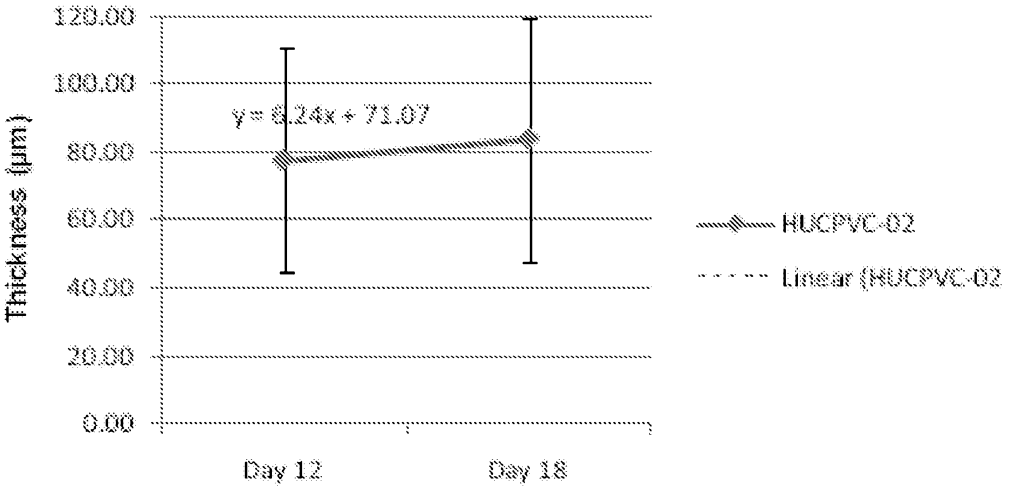

120.00
100.00
80.00
60.00
40.00
20.00
0.00
Thickness (µm)
y = 6.24x + 71.07
Day 12
Day 18
HUCPVC-02
Linear (HUCPVC-02

Figure 2

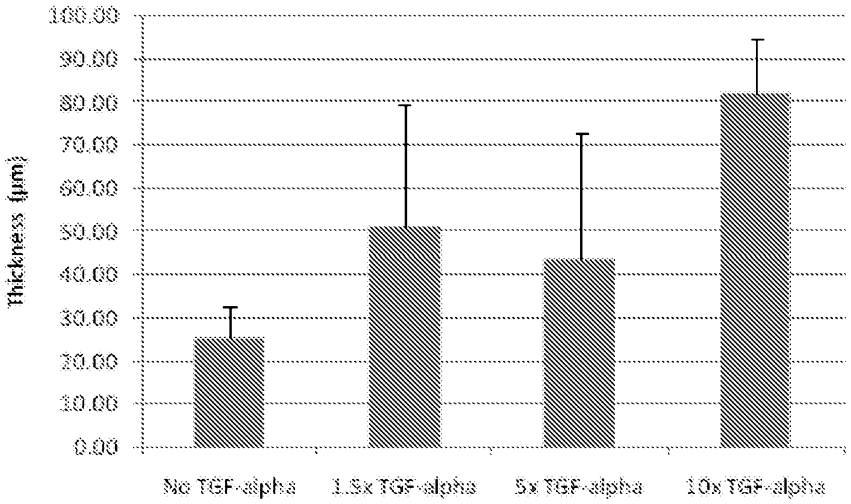


Figure 3

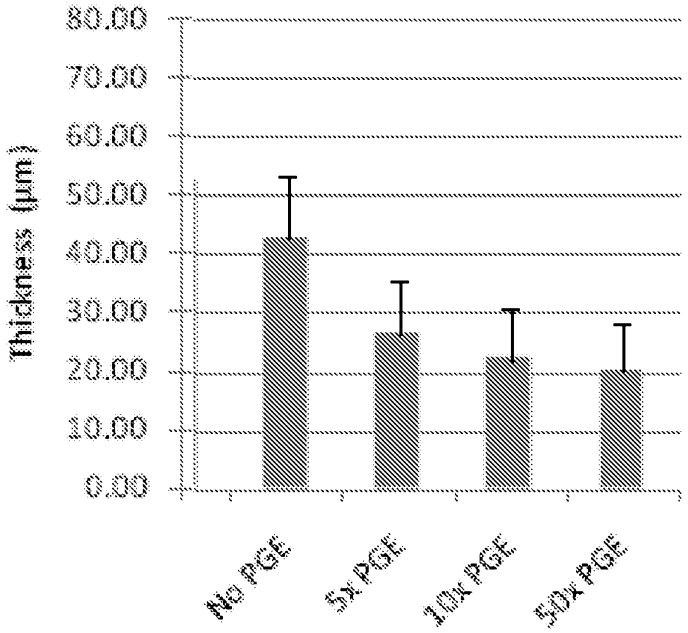


Figure 4

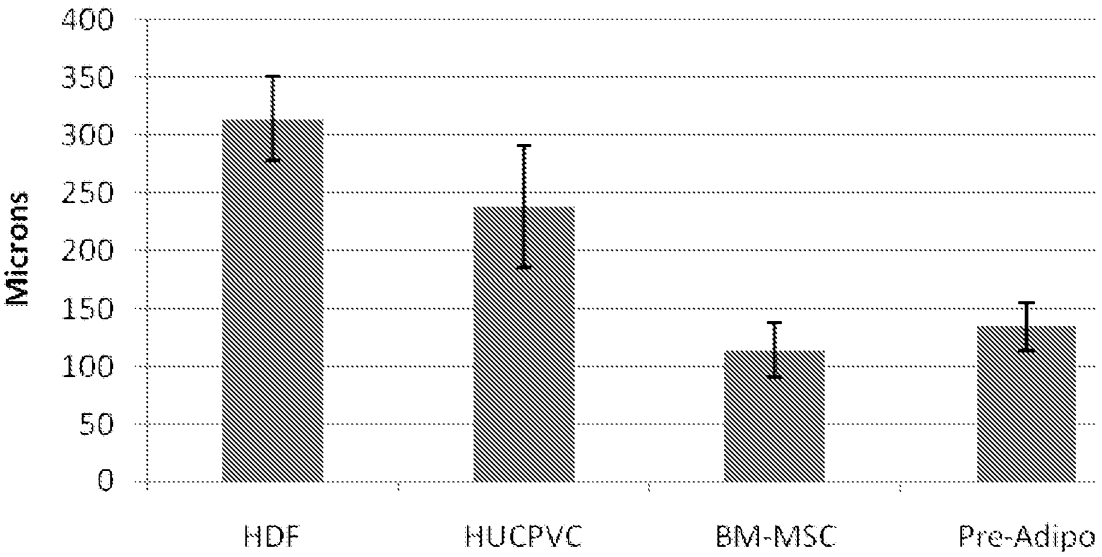
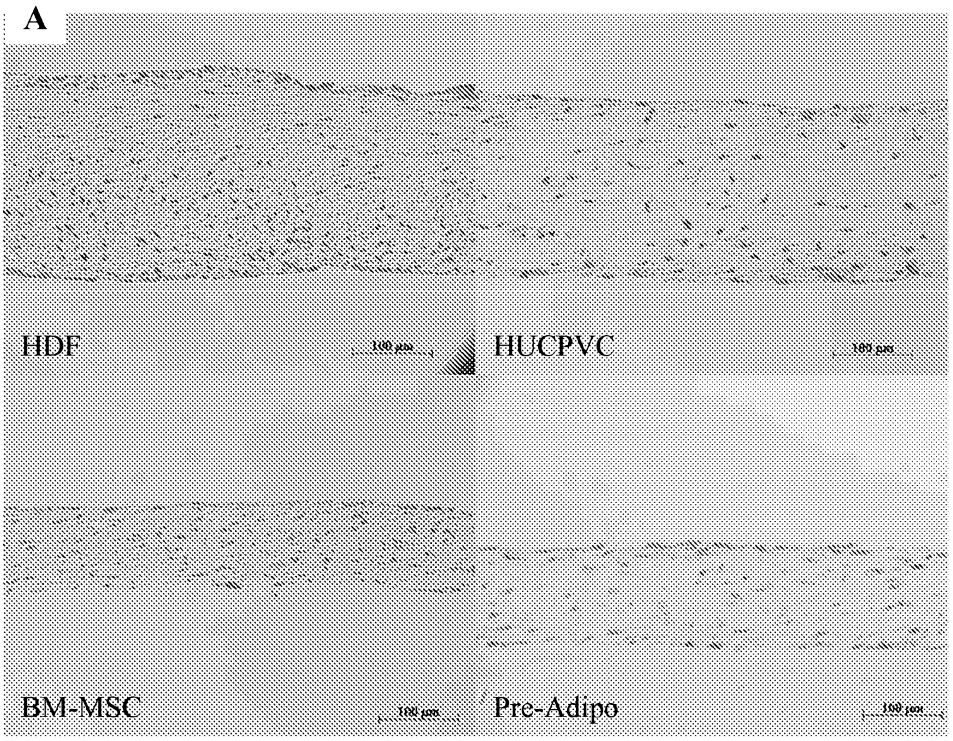

400
350
300
250
200
150
100
50
0
Microns
HDF
HUCPVC
BM-MSC
Pre-Adipo

Figure 5


A
HDF
HUCPVC
BM-MSC
Pre-Adipo

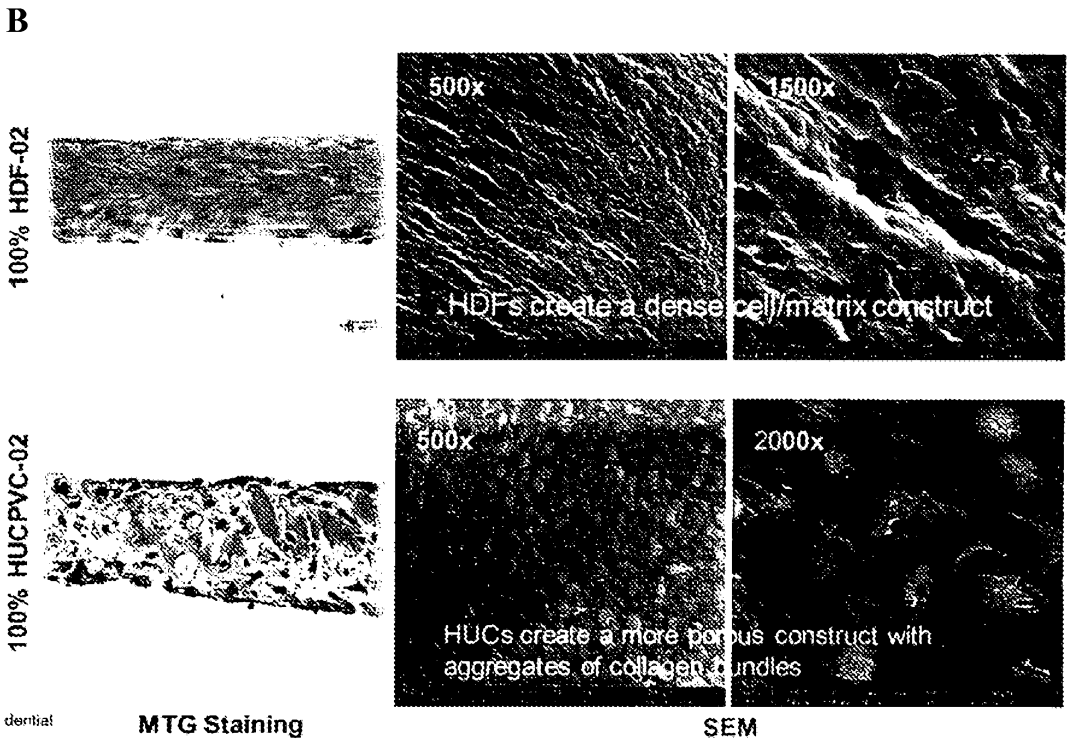
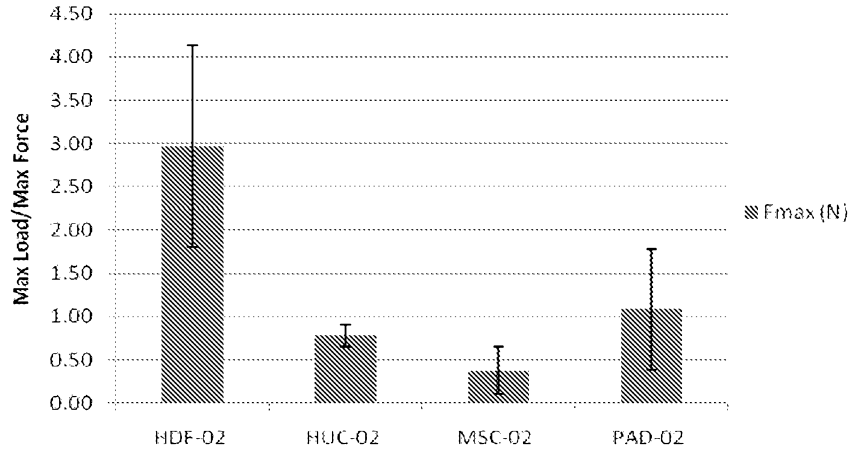
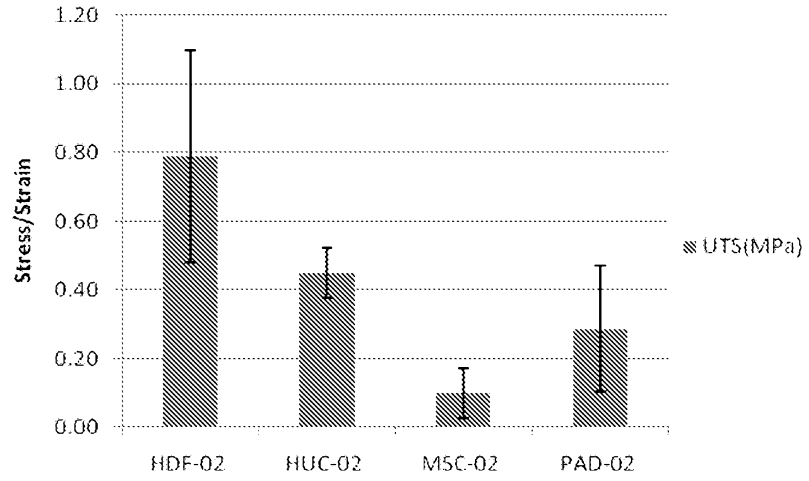

B
100% HDF-02
500x
1500x
HDFs create a dense cell/matrix construct
100% HUCPVC-02
500x
2000x
HUCs create a more porous construct with aggregates of collagen bundles
dermal
MTG Staining
SEM

Figure 6

A


4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Max Load/Max Force
Fmax (N)
HDF-02
HUC-02
MSC-02
PAD-02

B


1.20
1.00
0.80
0.60
0.40
0.20
0.00
Stress/Strain
UTS(MPa)
HDF-02
HUC-02
MSC-02
PAD-02

C

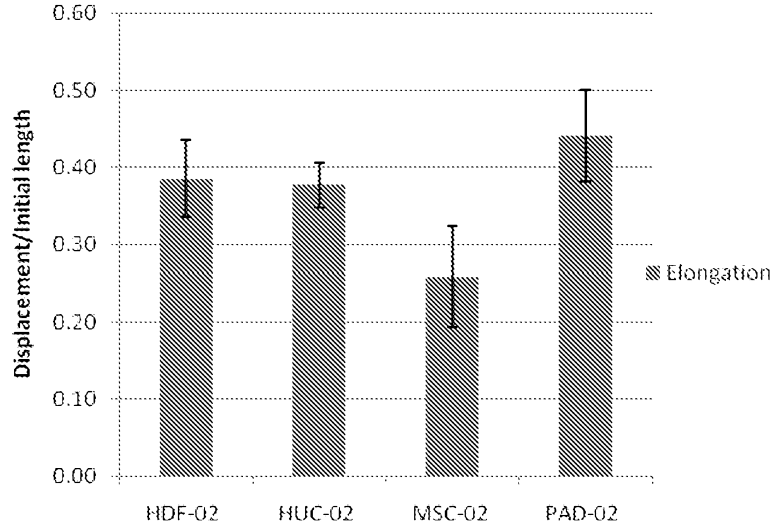
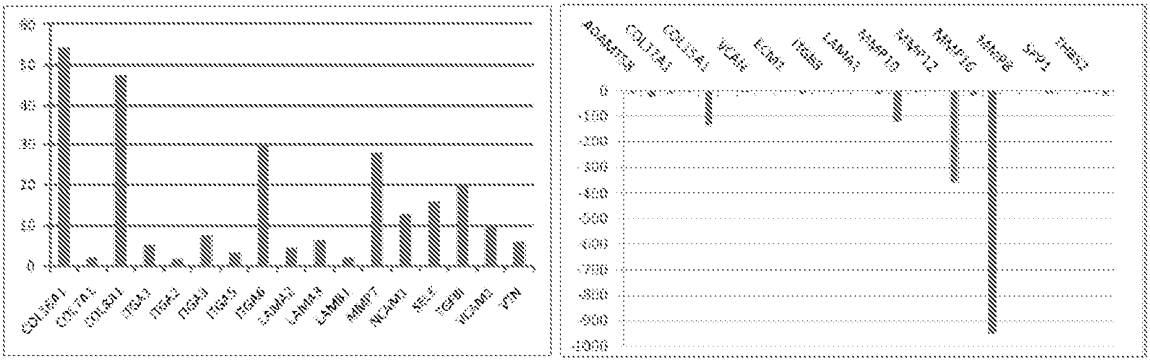

0.60
0.50
0.40
0.30
0.20
0.10
0.00
Displacement/Initial length
Elongation
HDF-02
HUC-02
MSC-02
PAD-02

Figure 7

A



B

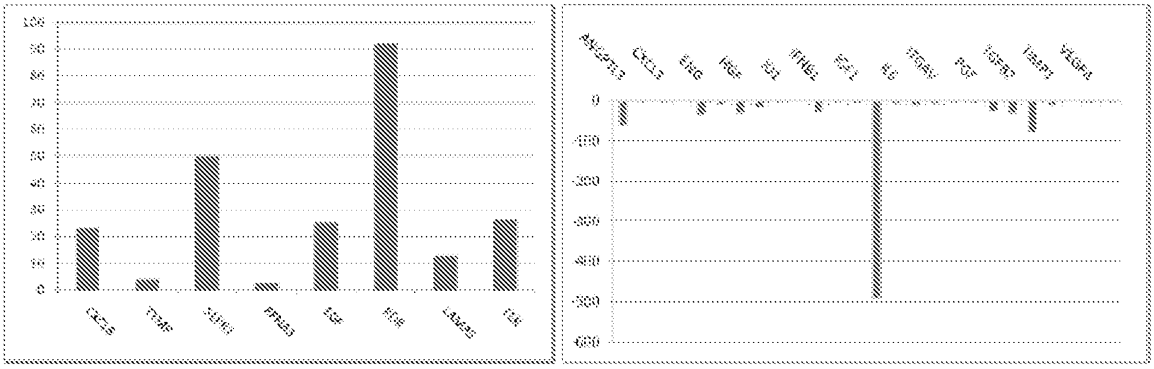
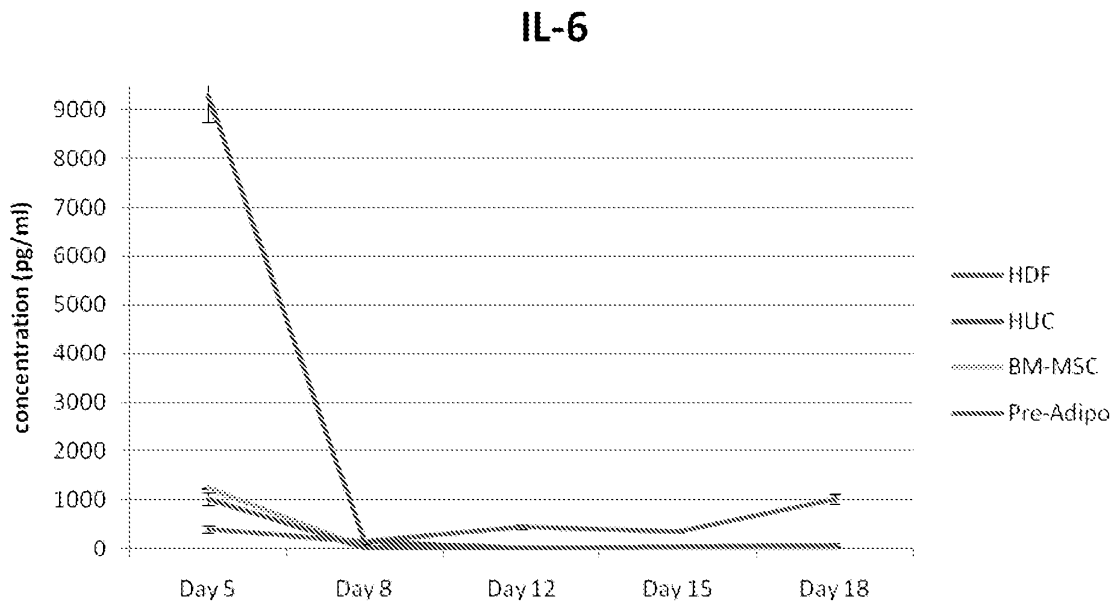


Figure 8

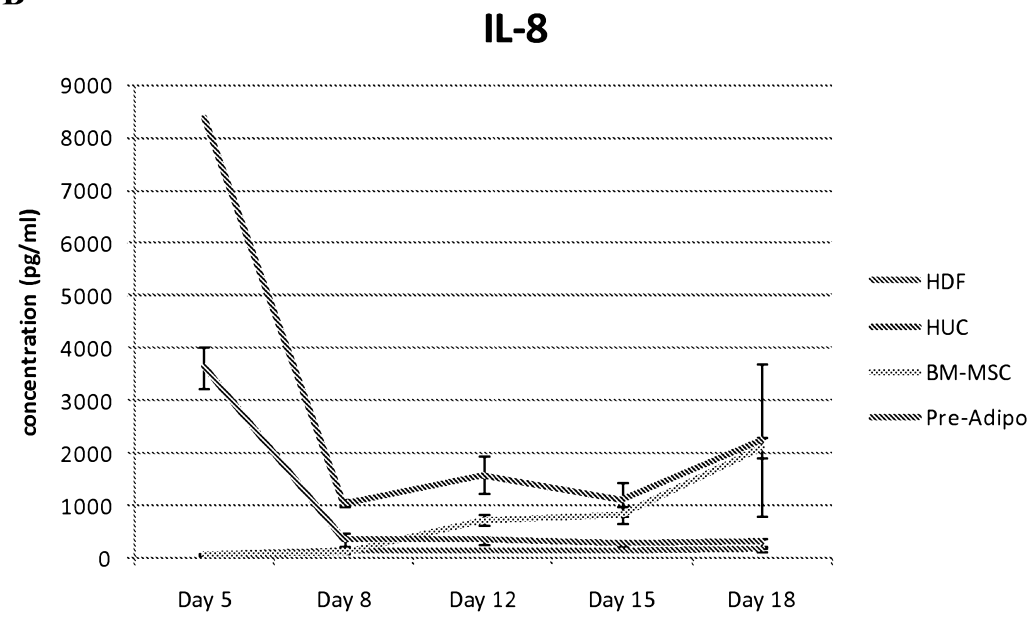
A



	Day 5		Day 8		Day 12		Day 15		Day 18	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
HDF	394.69	74.94	134.88	49.71	437.94	55.66	353.41	46.26	1020.66	108.56
HUC	9288.76	553.83	152.19	22.60	28.61	7.49	9.57	4.07	12.60	8.82
BM-MSC	1237.30	16.69	13.11	1.49	6.30	1.82	6.73	0.90	6.05	0.38
PAD	1018.62	122.30	12.32	46.36	12.32	2.71	38.29	13.12	71.85	39.23

Figure 8 (cont.)

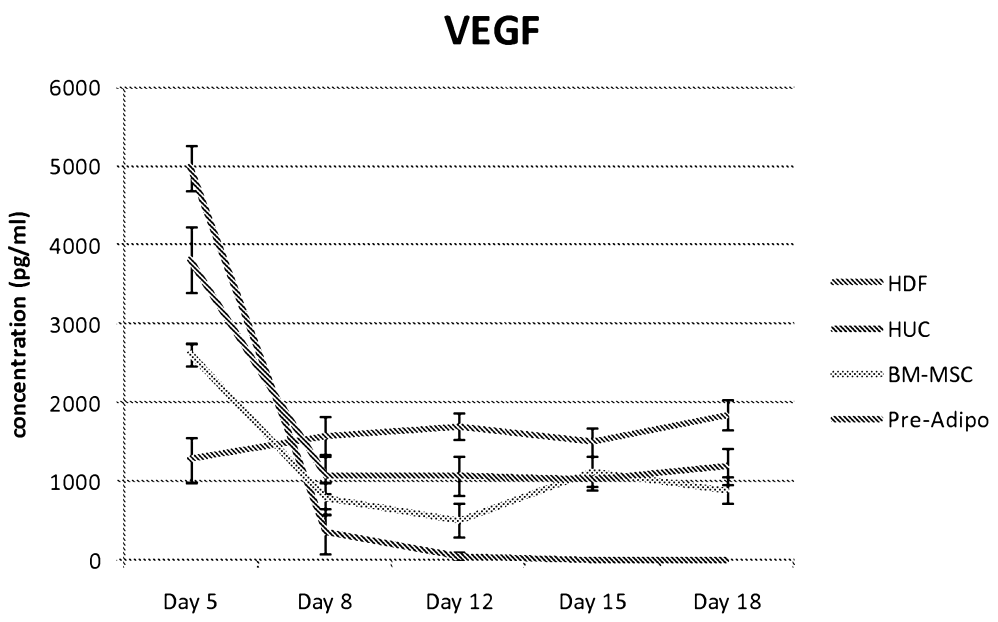
B



	Day 5		Day 8		Day 12		Day 15		Day 18	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
HDF	85.83	24.03	167.11	32.95	165.10	38.47	154.34	14.84	181.16	33.92
HUC	8386.27	0.00	1059.77	72.15	1589.65	357.67	1130.56	311.80	2247.12	1449.47
BM-MSC	81.73	13.16	115.97	28.91	736.53	96.94	840.62	155.40	2123.91	193.11
Pre-Adipo	3643.28	397.89	359.34	128.21	359.34	74.60	276.39	51.02	311.37	71.15

Figure 8 (cont.)

C



	Day 5		Day 8		Day 12		Day 15		Day 18	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
HDF	1280.51	285.55	1588.32	234.22	1701.42	165.45	1507.86	184.49	1846.74	191.80
HUC	4978.54	278.26	367.37	283.38	47.33	54.40	9.26	13.10	2.45	1.80
BM-MSC	2616.98	135.61	784.14	201.44	508.44	214.53	1118.02	213.38	890.62	169.78
Pre-Adipo	3815.19	416.97	1211.30	243.71	1083.62	253.49	1040.02	101.40	1196.41	233.68

Figure 8 (cont.)

D

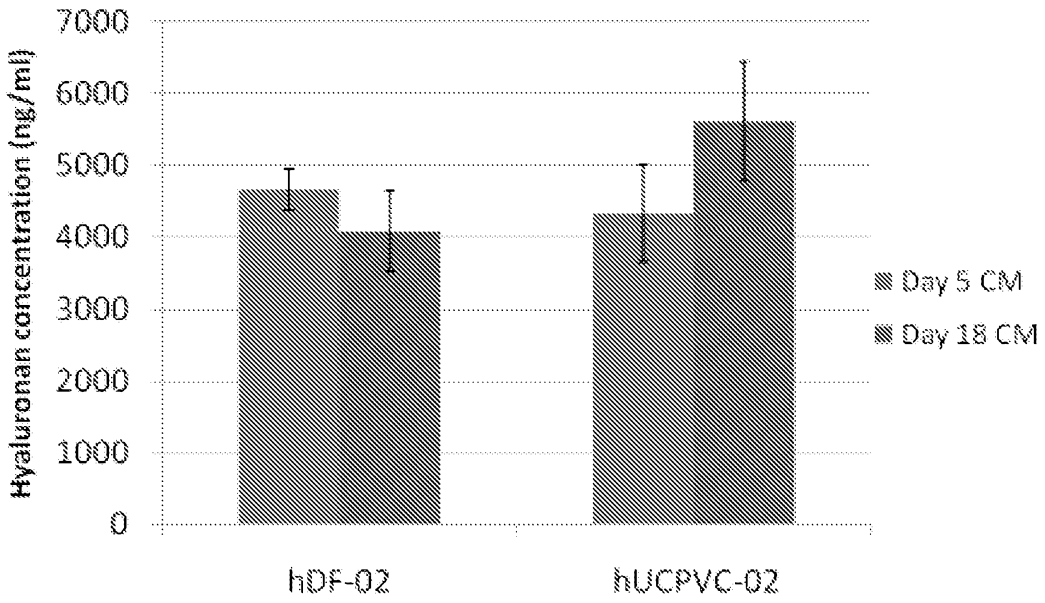


Figure 9

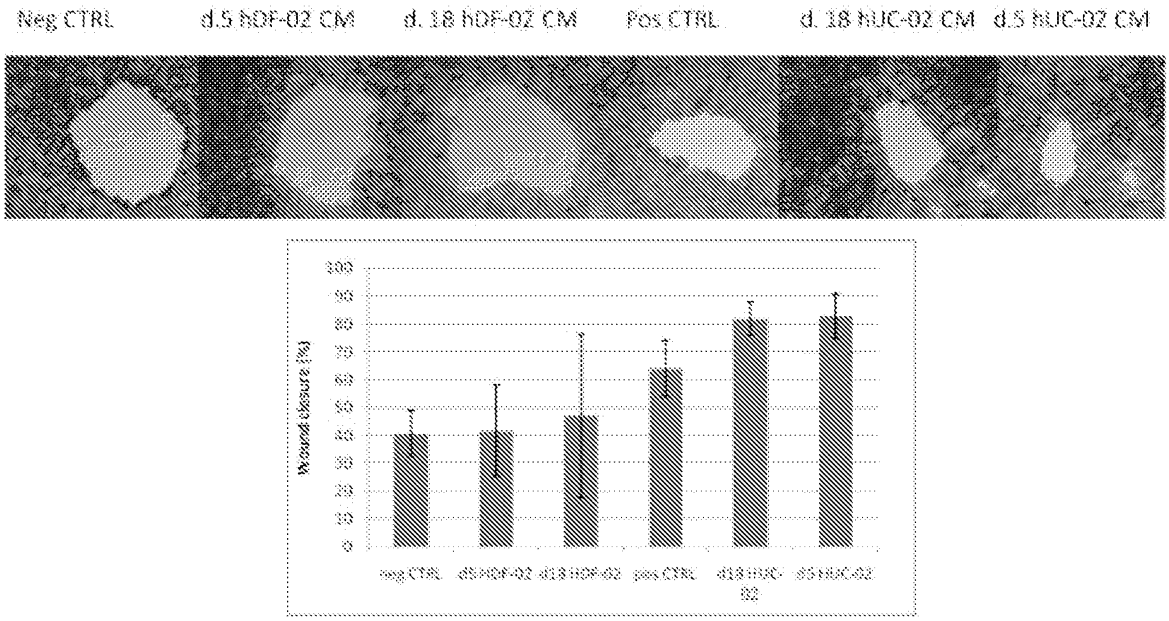

Neg CTRL
d.5 hDF-02 CM
d. 18 hDF-02 CM
Pos CTRL
d. 18 hUC-02 CM
d.5 hUC-02 CM
Wound closure (%)
0
10
20
30
40
50
60
70
80
90
100
neg CTRL
d5 hDF-02
d18 hDF-02
pos CTRL
d18 hUC-02
d5 hUC-02

Figure 10

A

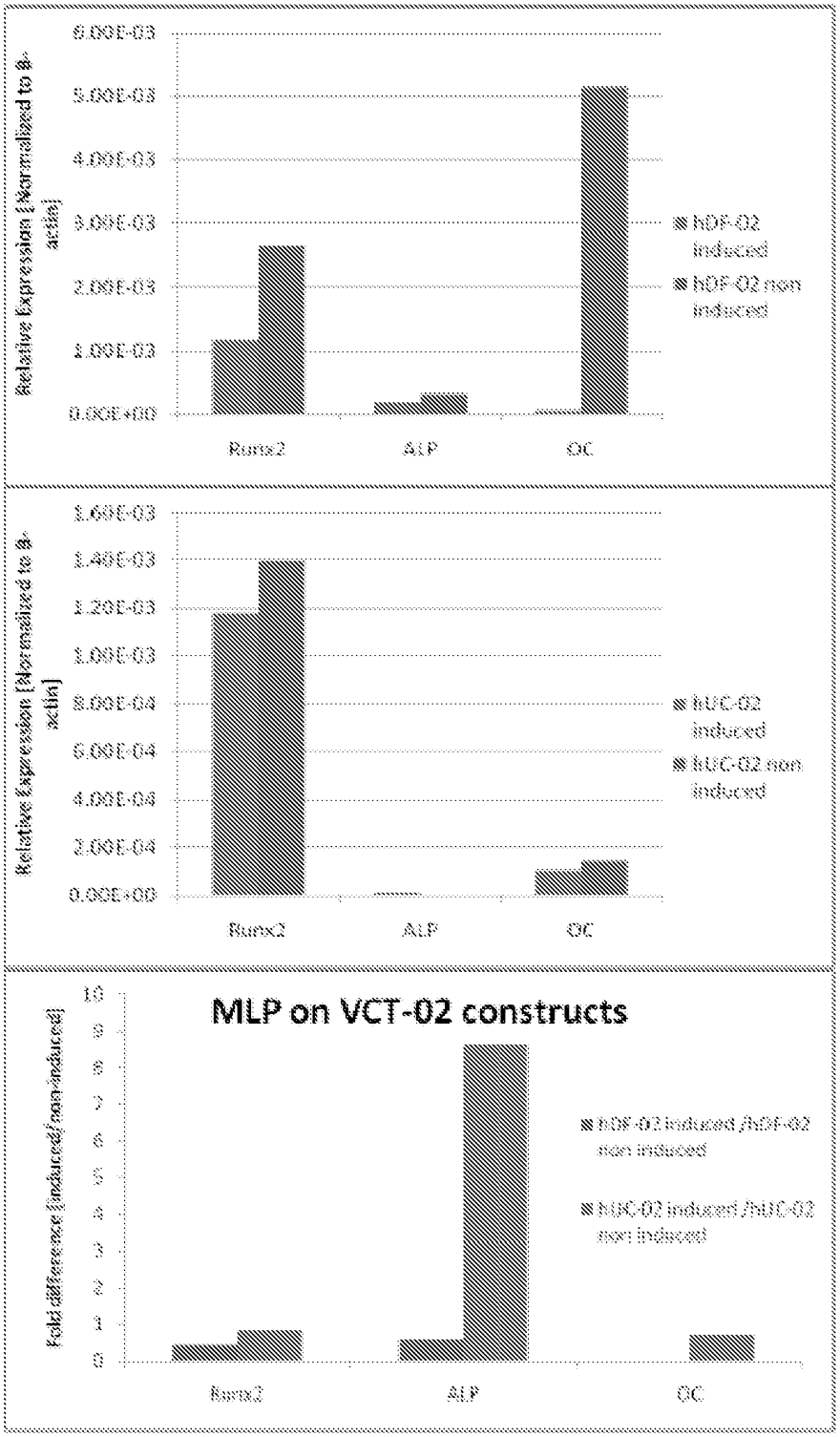

Relative Expression [Normalized to β-actin]
6.00E-03
5.00E-03
4.00E-03
3.00E-03
2.00E-03
1.00E-03
0.00E+00
hOF-02 induced
hOF-02 non induced
Runx2
ALP
OC
Relative Expression [Normalized to β-actin]
1.60E-03
1.40E-03
1.20E-03
1.00E-03
8.00E-04
6.00E-04
4.00E-04
2.00E-04
0.00E+00
hUC-02 induced
hUC-02 non induced
Runx2
ALP
OC
MLP on VCT-02 constructs
Fold difference [induced/ non-induced]
10
9
8
7
6
5
4
3
2
1
0
hOF-02 induced /hOF-02 non induced
hUC-02 induced /hUC-02 non induced
Runx2
ALP
OC

Figure 10 (cont.)

B

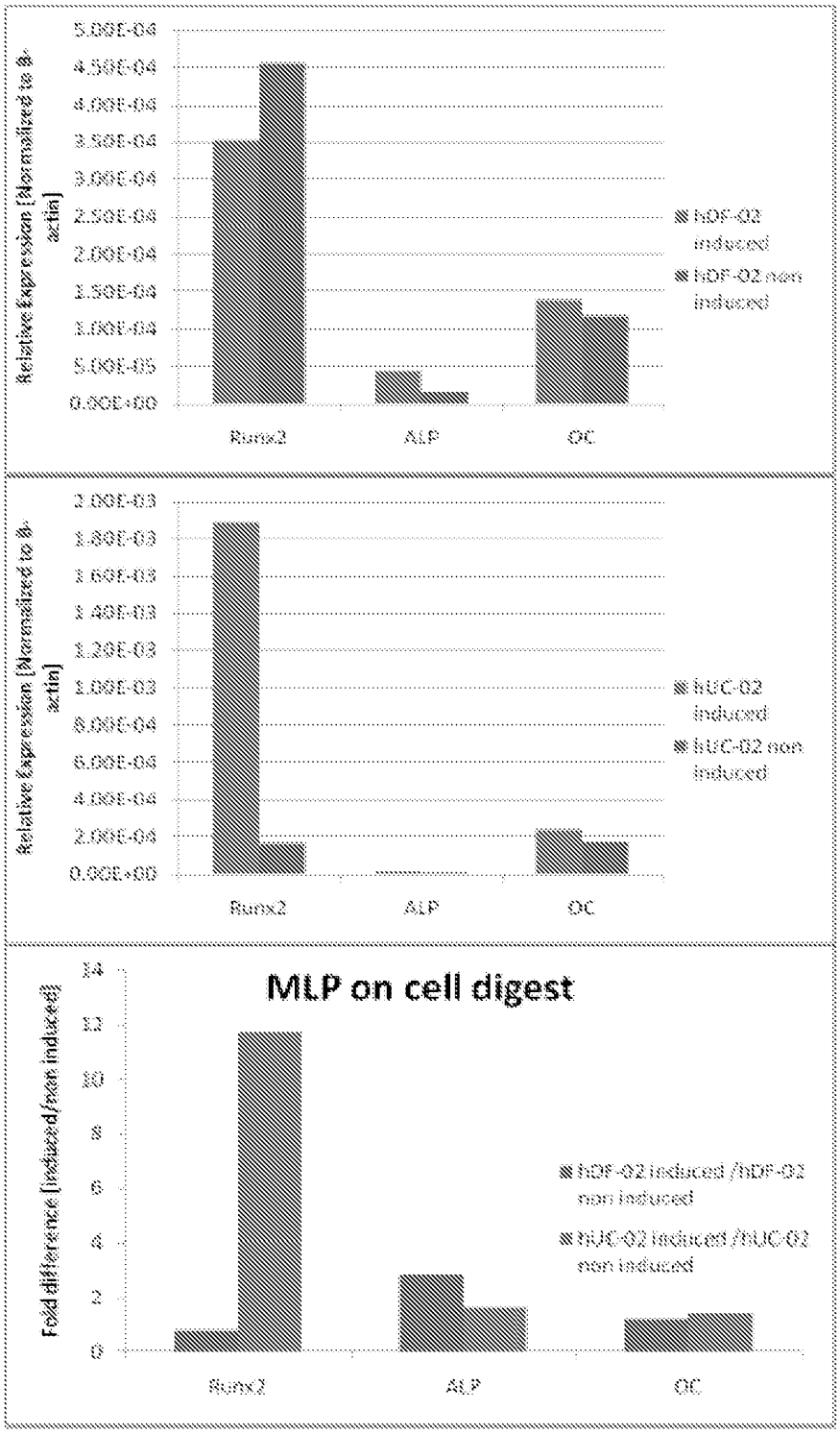


Figure 10 (cont.)

C

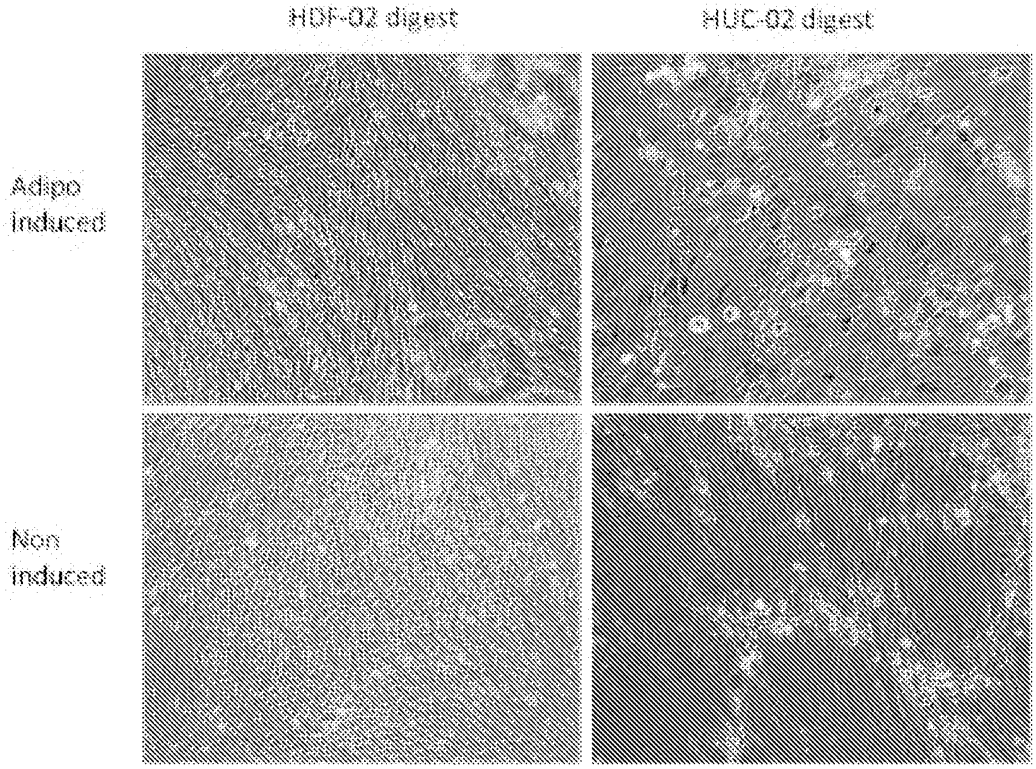
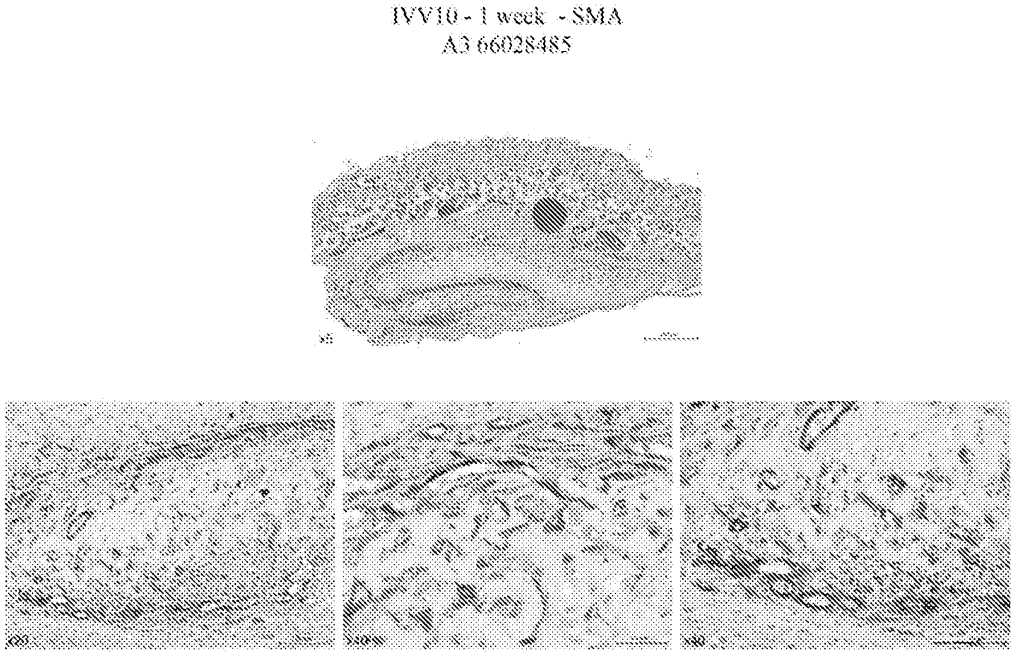


Figure 11

A



B

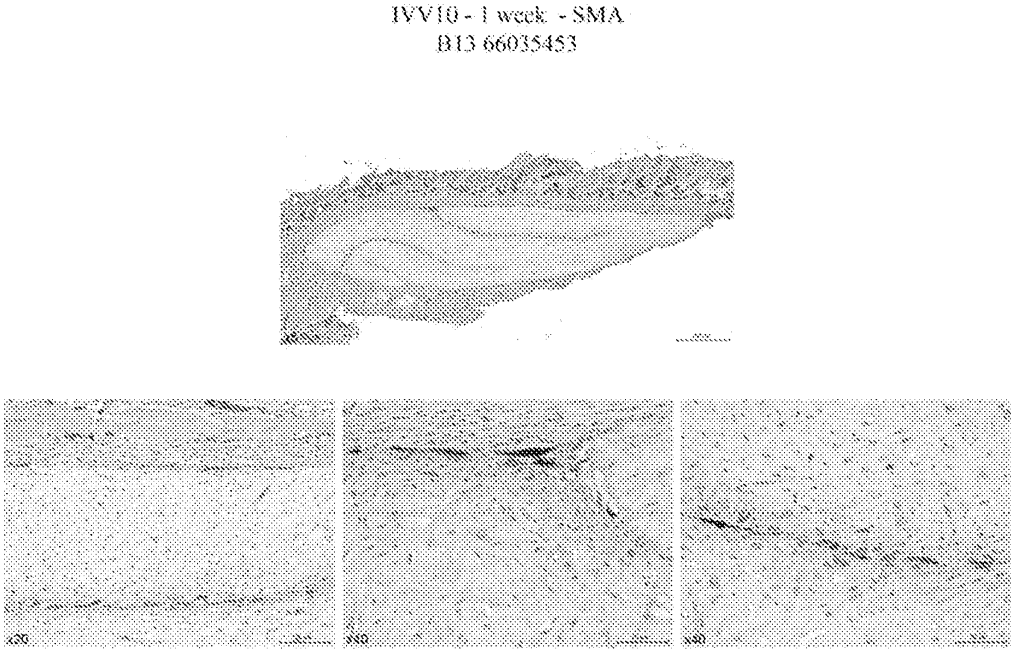
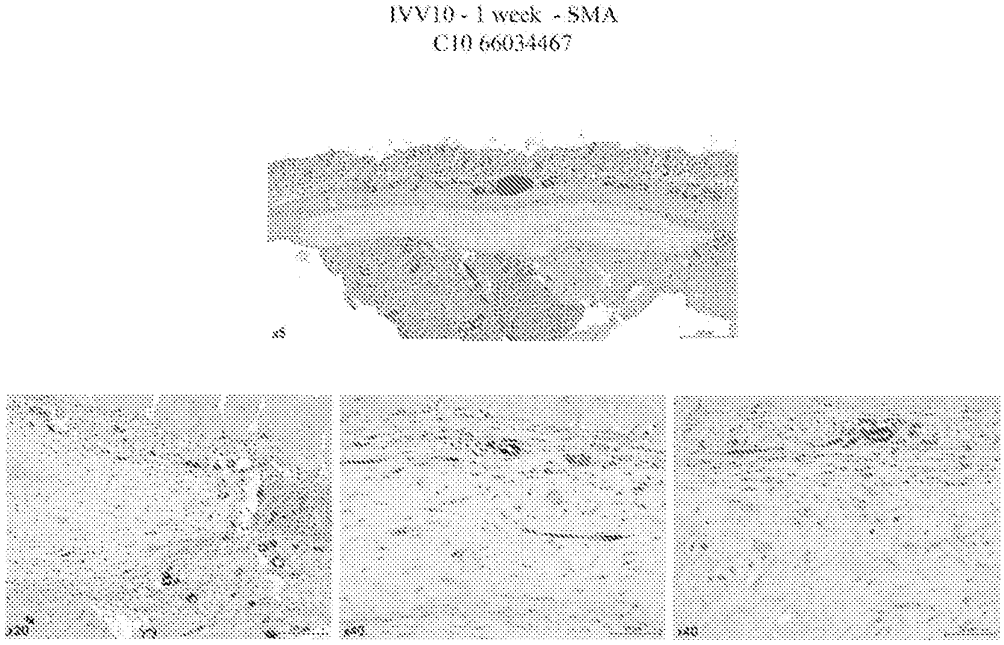


Figure 11 (cont.)

C



D

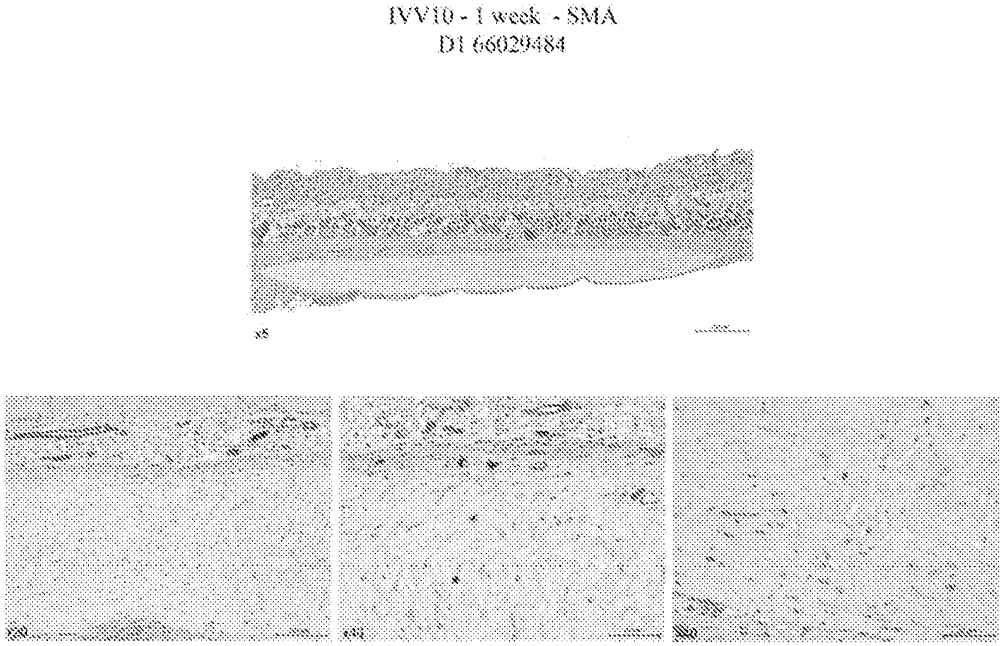
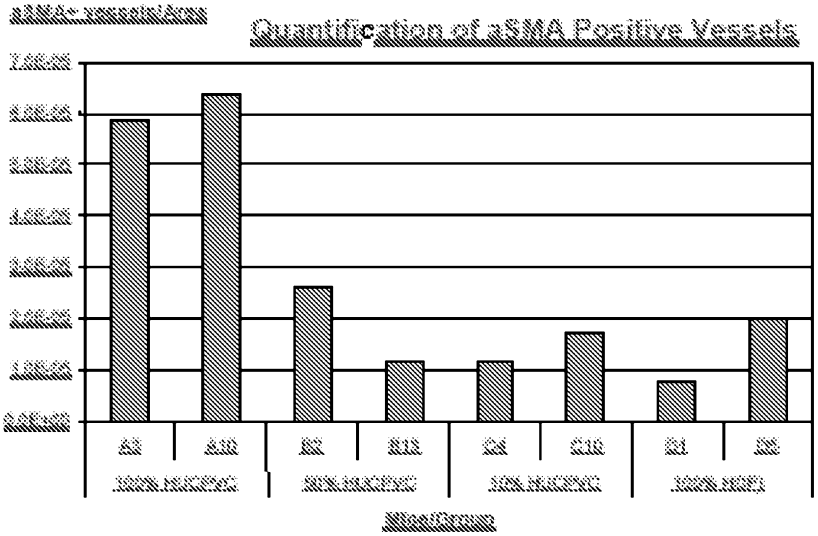


Figure 11 (cont.)

E



Group	Well	αSMA+ vessels/Area
100% HUCPVC	A3	3.3E-03
	A10	6.3E-03
50% HUCPVC	B2	1.6E-03
	B13	1.3E-03
10% HUCPVC	C4	1.1E-03
	C13	1.2E-03
100% HDF1	D1	8.0E-05
	D8	2.0E-03

Figure 12

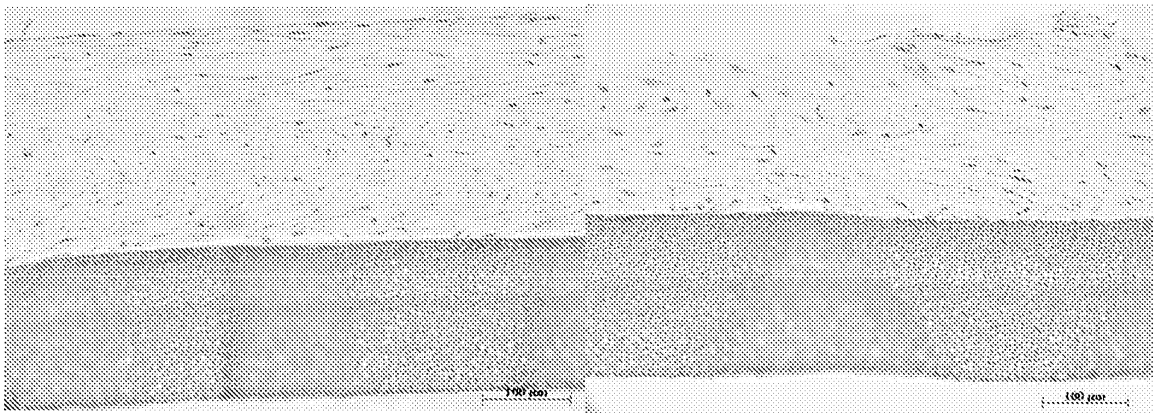


Figure 13

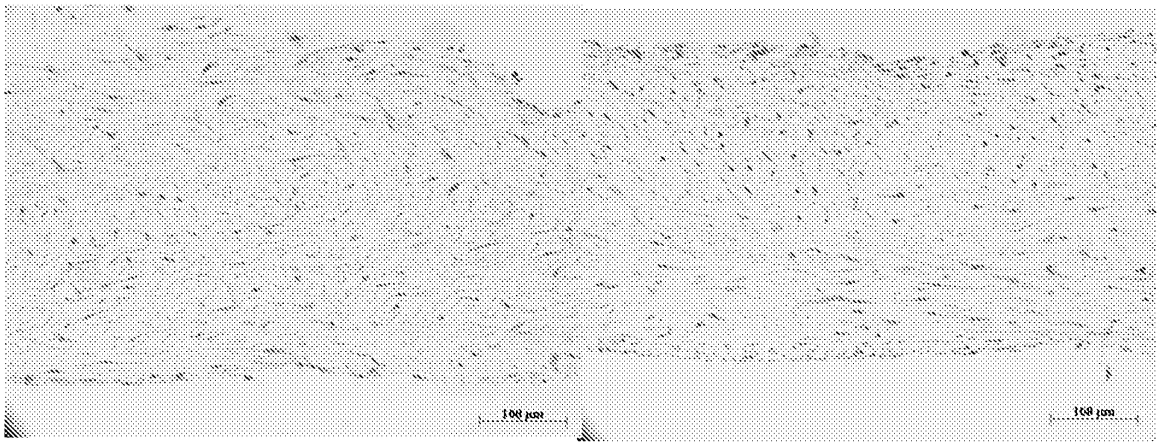
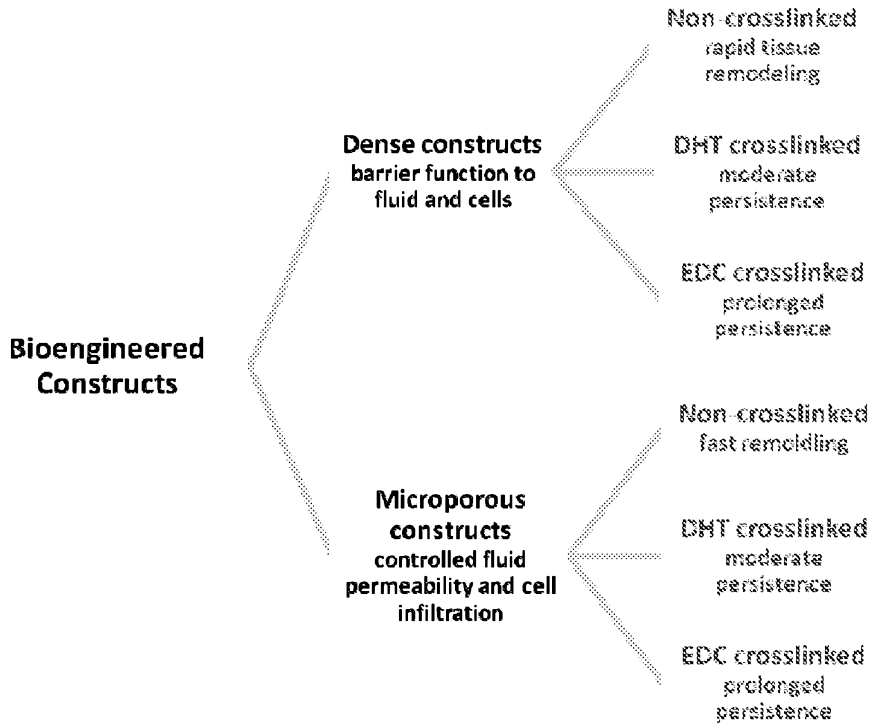


Figure 14

A



B

	Test A (EDC Crosslinked)	Test B (DHT Crosslinked)	Test C (Non-Crosslinked)
Avg.	16.675 μm	19.706 μm	18.569 μm
St.D.	7.467 μm	10.292 μm	8.984 μm
Min	4.869 μm	1.241 μm	1.423 μm
Max	40.865 μm	59.528 μm	71.128 μm

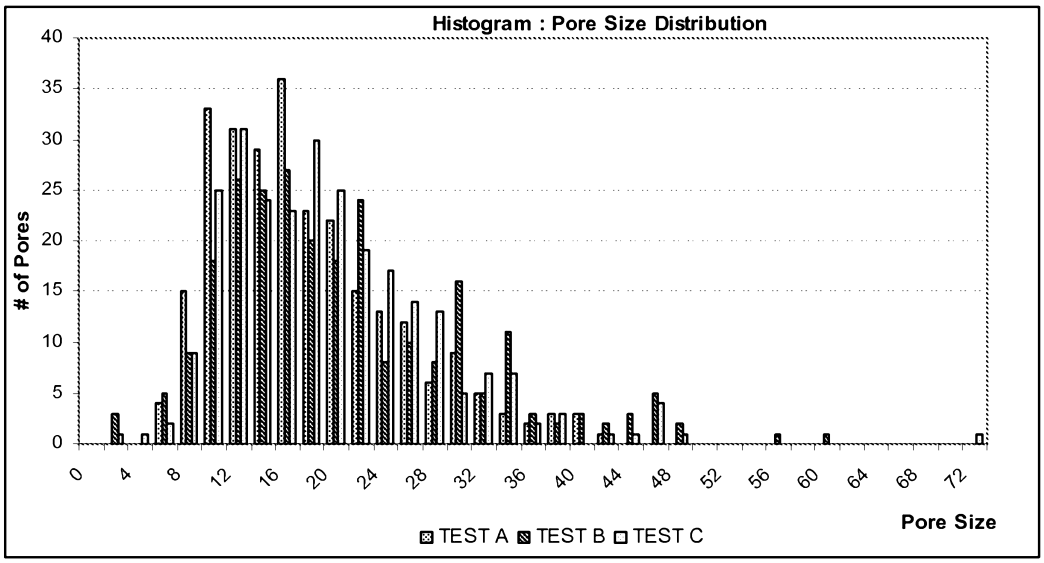
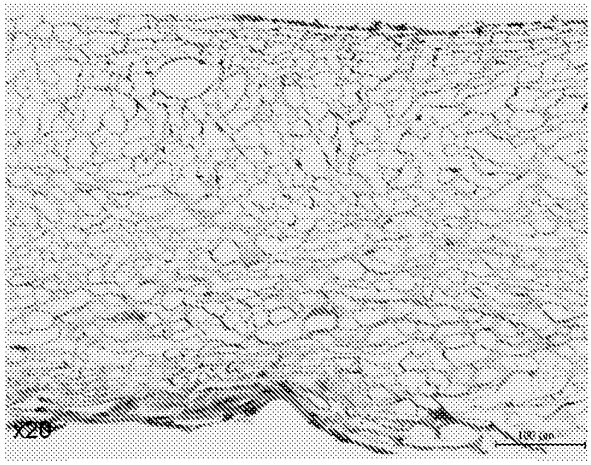


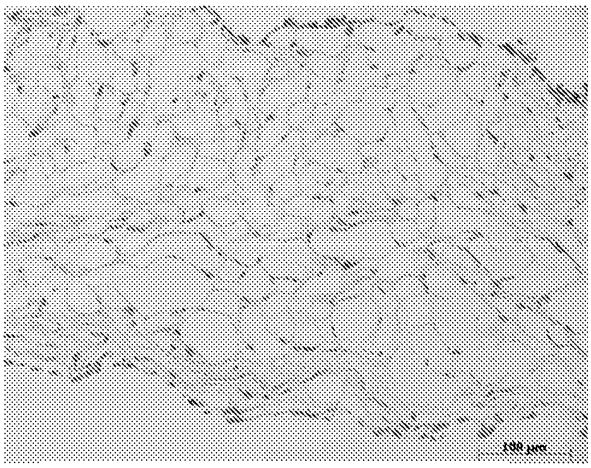
Figure 14 (cont.)

C



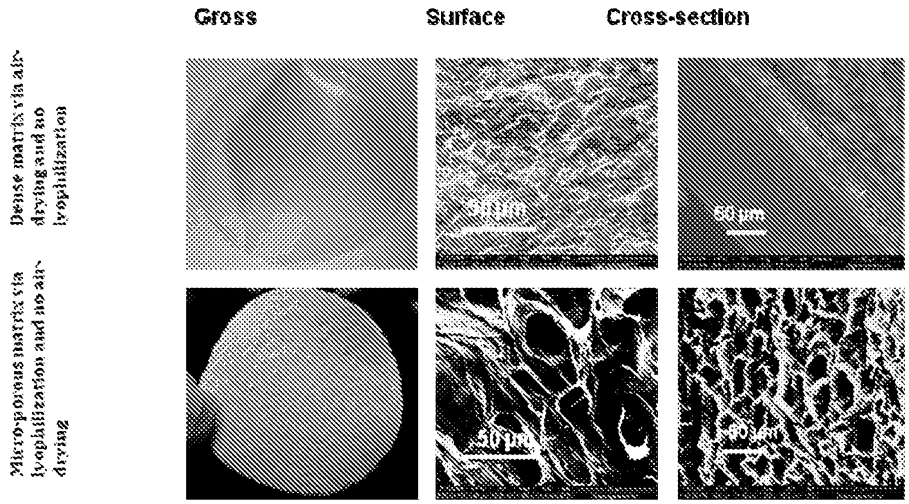
Average Pore Diameter: ~20 µm

D

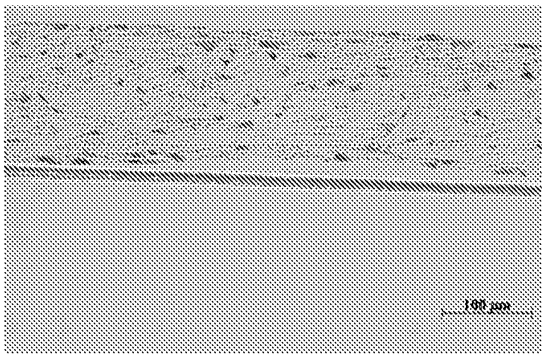


Average Pore Diameter: ~50 µm

E



F



G

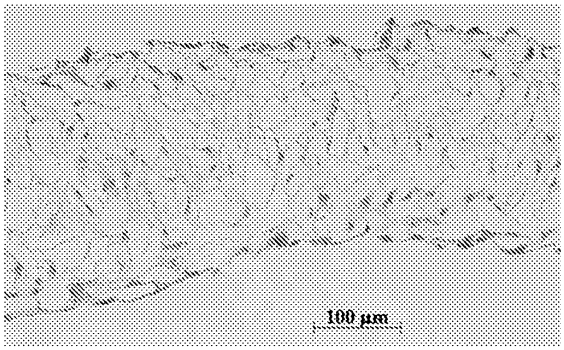
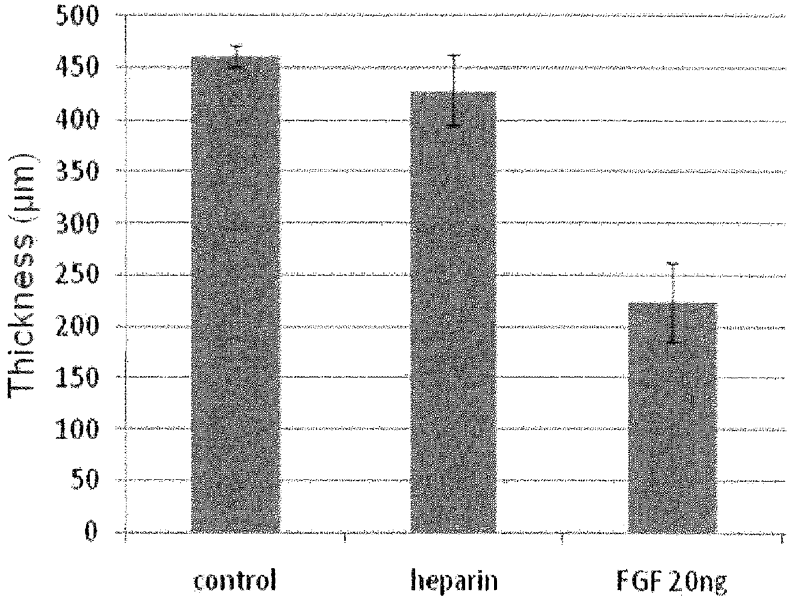


Figure 15

A


Thickness (μm)
500
450
400
350
300
250
200
150
100
50
0
control
heparin
FGF 20ng

B

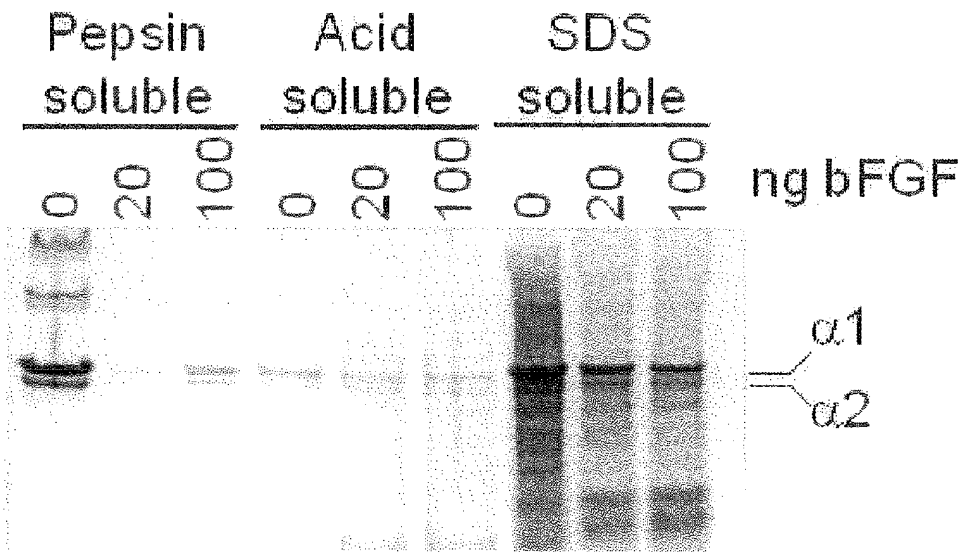
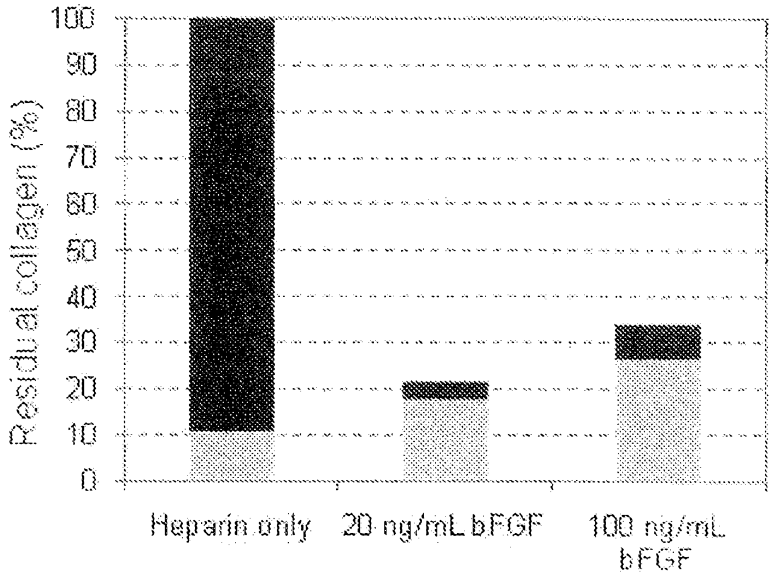
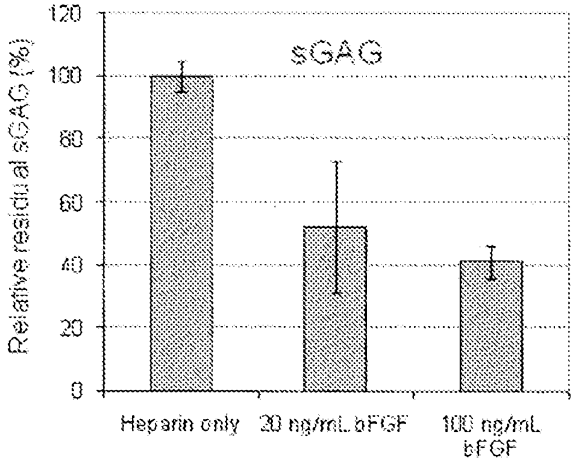

Pepsin soluble
Acid soluble
SDS soluble
0
20
100
0
20
100
0
20
100
ng bFGF
α1
α2

Figure 15 (cont.)

C



D



E

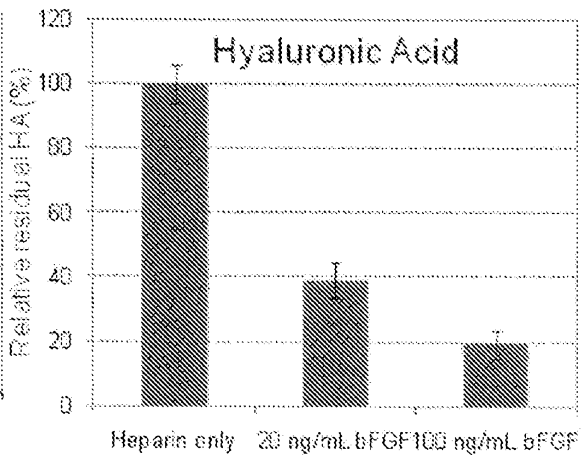


Figure 16

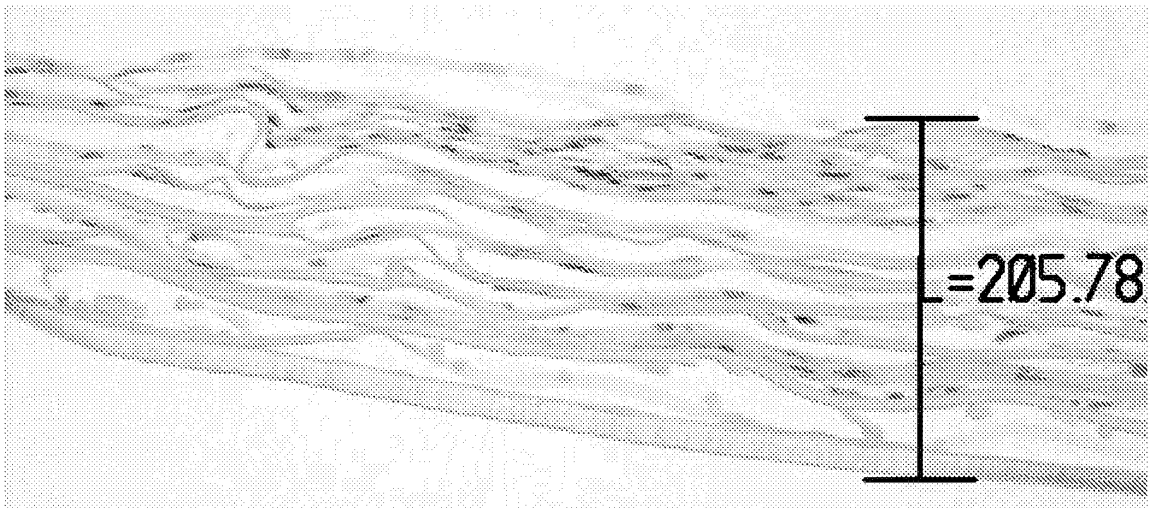
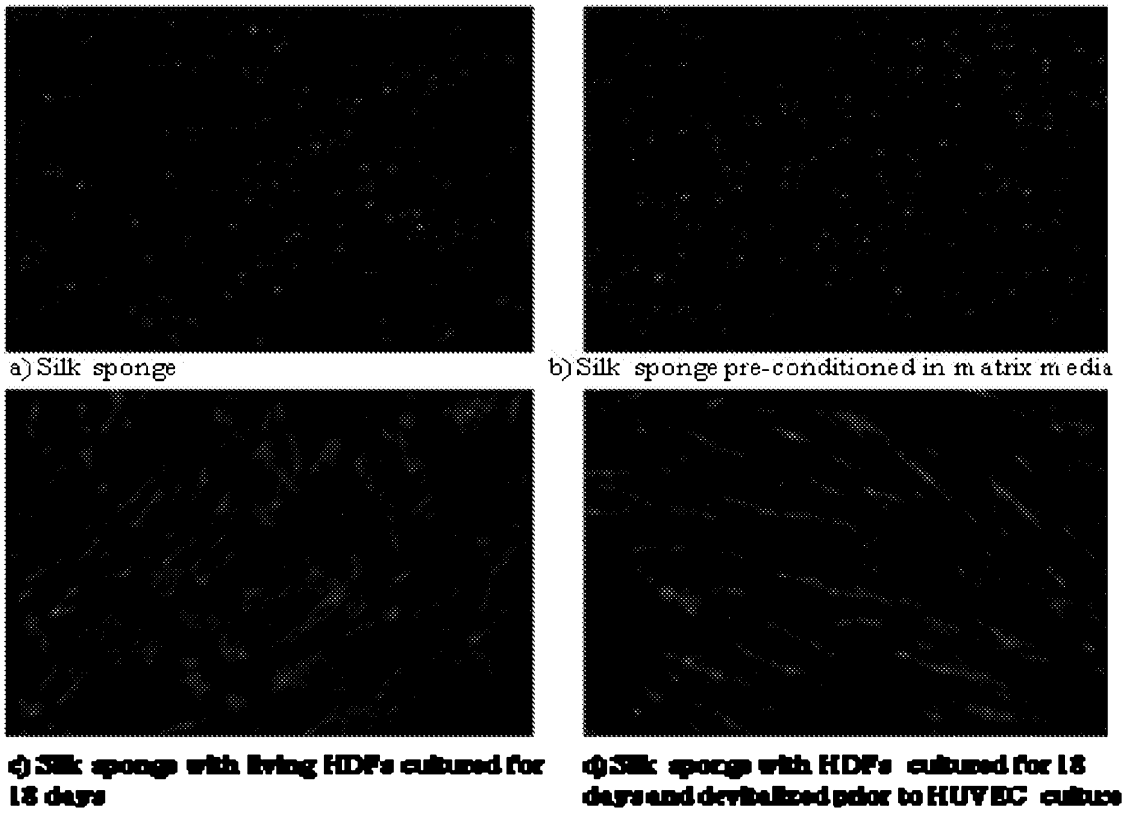


Figure 17


a) Silk sponge
b) Silk sponge pre-conditioned in matrix media
c) Silk sponge with living HDFs cultured for 18 days
d) Silk sponge with HDFs cultured for 18 days and devitalized prior to HUVEC culture