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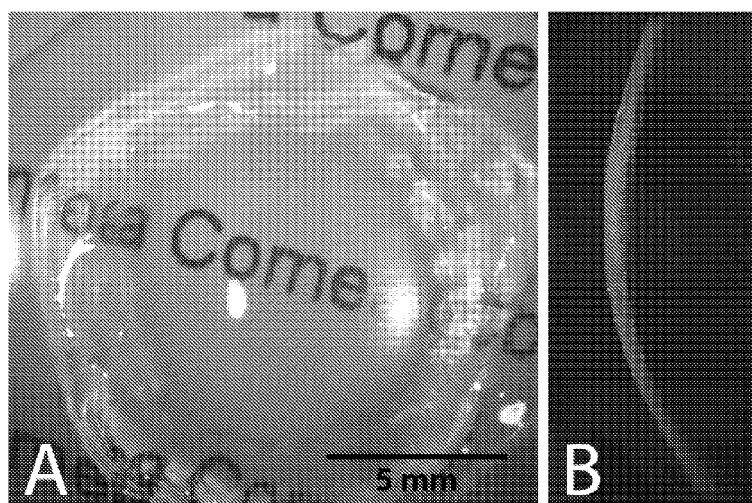
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(54) **Title:** COLLAGEN COMPOSITIONS AND PREPARATION AND USES THEREOF

Figure 1



(57) **Abstract:** The present invention encompasses compositions comprising collagen that may be used to repair or augment various tissues of the body, including eye tissues, and particularly, the tissues of the cornea. Also encompassed are methods of making and utilising these compositions.

COLLAGEN COMPOSITIONS AND PREPARATION AND USES THEREOF

RELATED APPLICATION

[0001] This application claims the benefit of New Zealand provisional patent application number 707680, filed 1 May 2015, the entire contents of which are incorporated by reference herein.

FIELD OF THE INVENTION

[0002] The present disclosure relates to compositions and methods useful for the treatment and/or prevention of conditions of the eye, amongst other bodily conditions. In particular, the disclosure relates to compositions comprising collagen and methods of making and using these compositions. Specifically disclosed are methods for augmenting and repairing eye tissue, in particular, corneal tissue, as well as other epithelial tissue, and also muscle, nervous, and connective tissue.

BACKGROUND OF THE INVENTION

[0003] Corneal transplantation remains the gold standard therapy for diseases that cause corneal blindness (Tan et al., 2012). In the United States, 72,736 corneal transplants were performed in 2013, up 5.9% from 2012 (Eye Bank Association of America, 2013). However, cadaveric donor tissue is a limited resource in most countries and unavailable in some, with demand increasingly exceeding supply (Tan et al., 2012). There is also the problem of graft rejection. Between 5% and 30% of grafts will fail within 10 years, and failure rate is affected by various risk factors (Sugar et al., 2009).

[0004] Although artificial corneas (keratoprotheses) such as the Boston KPro or AlphaCor are available for clinical use, they suffer a number of limitations. Common complications of keratoprotheses include formation of a retro-prosthetic membrane, extrusion, chronic inflammation, infection, melting at the cornea prosthesis interface, and leakage (Shihadeh and Mohidat, 2012; Hassanaly et al., 2014; Kim et al., 2013; Lekhanont K, et al., 2014; Magalhaes et al., 2013).

[0005] The ideal corneal tissue substitute should be transparent and biocompatible, closely resemble the structure of the cornea, have similar elasticity and tensile strength to that of native tissue (thereby enabling surgical manipulation), allow the diffusion of nutrients, induce minimal inflammatory response, and support cell attachment, proliferation, and differentiation (Yang et al., 2001; Lalan et al., 2001; Carlsson et al., 2003; Myung et al., 2008; Lynch and

Ahearne, 2013). Transparency (high optical transmittance), uniform refractive index, and maintenance of shape are also essential characteristics (Maurice, 1957).

[0006] Scaffolds with synthetic constituents may meet the biomechanical demands required, but they do not readily support cell attachment, proliferation and differentiation (Yang et al., 2001). Researchers have therefore attempted to produce tissue substitutes using biological components. Collagen is the major protein constituent of the extracellular matrix and so has been a commonly used component in tissue scaffold construction (Krieg et al., 1988). Collagen materials have been utilised for tissue engineering for the cornea and other body tissues, including bone, cartilage, tendon, skin, urethral, bladder, heart, and venous tissues (see, e.g., Parenteau-Bareil et al., 2010). Scaffolds comprised of collagen have been used, particularly, in tissue regeneration, and in the treatment of wounds and burns (Oliviera et al., 2009; Singh et al., 2011). Collagen scaffolds have been typically formed as sponges, thin sheets, or gels, and have been formulated to include other components, such as chitosan (Berillo et al., 2012). The significant drawbacks for collagen scaffolds have been their degradation and inability to withstand mechanical forces (see, e.g., Parenteau-Bareil et al., 2010).

[0007] Collagen molecules consist of three polypeptide chains (α chains) that form a triple helix. These rod-shaped molecules are approximately 3000 Å in length and 15 Å in diameter and have a molecular weight of approximately 300 kDa (Streeter and de Leeuw, 2010). Self-assembly of collagen into fibrils has been observed in purified soluble collagen solutions *in vitro*. This occurs when the temperature pH, and/or the ionic strength are increased to optimal levels (Williams et al., 1978). However, the resulting hydrogels tend to have relatively low collagen concentrations and consist of randomly organised collagen fibrils (Hwang and Lyubovitsky, 2011; Helary et al., 2010). These gel matrices also suffer the disadvantage of very poor tensile strength, with reported Young's Modulus in the order of kilo Pascals (kPa) (<http://www.advancedbiomatrix.com>, 2010; Ahearne et al., 2008; Osborne et al., 1998). The tensile strengths of collagen sponges are similarly low (Borene et al., 2004; Zhang et al., 2013).

[0008] Methods involving evaporation, lyophilisation, and dialysis have been used to produce concentrated collagen solutions. Evaporation of dilute collagen solutions in an open dish has been used to produce concentrated collagen solutions (Helary et al., 2005). However, this technique requires large volumes of dilute collagen to be placed in a container with a large diameter and small height to minimise the evaporation time. An alternative technique continuously injected dilute collagen solutions into glass microchambers as evaporation proceeded (Mosser et al., 2006). Yet, this technique takes weeks to produce a concentrated

solution. In both cases, the resulting solutions have a concentration gradient, which prevents uniform and consistent scaffold formation.

[0009] Lyophilised collagen is readily available and may be used to produce collagen solutions by adding acid and stirring for a period of hours. However, concentrated collagen solutions are highly viscous, making it difficult to obtain homogenous solutions. Additionally, the stirring action disrupts the organisation of the liquid crystalline solution.

[0010] In methods employing dialysis, large initial volumes of collagen solution are required to produce a small volume of sufficiently concentrated collagen (e.g., 15 ml of 3 mg/ml collagen concentrated to 0.15 ml of 300 mg/ml collagen). Since the rate of dialysis is directly proportional to the surface area of the membrane, a large surface area of dialyzing membrane is usually required. Moreover, the concentrated collagen forms in a thin layer spread across the whole surface area of the membrane. Scraping is therefore required to remove the concentrated collagen, thereby disrupting the organisation of the liquid crystalline solution. This disrupts any organised structure formed by the collagen. Further, if a small surface area of dialyzing membrane is used, dialysis occurs at a very slow rate and as the layer of concentrated collagen builds up, the rate of dialysis slows due to clogging of the membrane. Despite the use of membranes with very fine pores, it is effectively impossible to achieve 100% retention of even very large molecules. Use of dialysate molecules (e.g., polyethylene glycol) is thus required.

[0011] Plastic compression of collagen hydrogels has also been used to increase the collagen concentration (WO 2006/003442). Young's modulus for these constructs has been reported to be approximately 1800 kPa (Hadjipanayi, 2009). Although the collagen fibrils within these constructs are more densely packed than in collagen hydrogels, they remain randomly oriented (Mi et al., 2010). Transparency has been reported to be 66% at 550 nm, (Levis et al., 2013), and the surface of the construct is imprinted with the pattern of the nylon mesh used for compression (Alekseeva et al., 2012). This technique is further limited in that only flat constructs of limited thickness can be produced. Therefore, plastic compressed collagen does not meet the demands required of a corneal tissue substitute.

[0012] As well as determining tensile strength, the precise spatial arrangement of collagen *in vivo* plays an important role in cell signalling, and directing proliferation and migration (Lee et al., 2006). Previous attempts at producing aligned collagen scaffolds have used techniques such as electrospinning (Matthews et al., 2002), magnetic field alignment (Girton et al., 1999), and shear flow (Lanfer et al., 2008). Electrospun collagen scaffolds are limited in thickness (typically less than 1 mm) and precise control of where the fibres are deposited is not possible.

Additionally, fibre morphology varies from scaffold to scaffold. Magnetic field alignment is expensive due to the requirement for a high magnetic field (approximately 7 Tesla). Although an effective method of aligning collagen, only low concentration hydrogels have been produced with this method. Using shear flow to induce alignment limits production to thin scaffolds of less than 50 μm thickness.

[0013] Therefore, there is an ongoing need for therapeutic compositions, in particular, compositions comprising collagen, and methods for addressing tissue disease or damage, including methods for the repair and/or augmentation of eye tissue, as well as other tissues. There is a particular need for therapeutic approaches that are efficacious and readily utilised.

SUMMARY OF THE INVENTION

[0014] The inventors have developed collagen compositions and methods of preparing and employing these compositions. The compositions and methods are useful for repairing and/or augmenting eye tissue, along with other tissues of the body, and thereby treating and/or preventing various conditions of eye and other bodily conditions.

[0015] In one aspect, the invention encompasses a method of preparing a collagen composition, the method comprising:

- a) obtaining a collagen gel having a collagen concentration of 100 mg/ml to 190 mg/ml;
 - b) hydrating the collagen gel such that liquid content is increased 10-fold to 20-fold;
- and,
- c) combining two or more aliquots of hydrated collagen gel such that a combined gel aliquot of increased thickness is obtained.

[0016] In various aspects:

[0017] The method further comprises: moulding the combined gel aliquot.

[0018] The method further comprises: neutralising the pH of the combined gel aliquot.

[0019] The method further comprises: heating the combined gel aliquot under humidity, thereby allowing the gel to set.

[0020] The method further comprises: dehydrating the set gel to completion.

[0021] The method further comprises: rehydrating the dehydrated gel by immersion in liquid.

[0022] The method further comprises: crosslinking the combined gel aliquot following the dehydration and rehydration steps.

[0023] The concentration of (a) is obtained by centrifugation and ultrafiltration of a collagen solution.

- [0024] The concentration of (a) is obtained by concentrating a collagen solution 10-fold to 50-fold by centrifugation in combination with ultrafiltration.
- [0025] The hydrating of (b) is carried out by centrifugation of the collagen gel in the presence of the liquid.
- [0026] The collagen gel is compressed prior to (b).
- [0027] The combining of (c) is carried out by centrifugation of the two or more aliquots of hydrated collagen gel.
- [0028] The moulding step is carried out at the same time as the heating step.
- [0029] The neutralising step is carried out prior to or at the same time as the heating step.
- [0030] The collagen solution is 3 mg/ml to 10 mg/ml.
- [0031] The collagen solution comprises collagen provided in an acetic acid solution.
- [0032] The ultrafiltration is carried out with a molecular weight cut-off of 10 kDa.
- [0033] The centrifugation is carried out at 2000 x g to 4000 x g.
- [0034] The centrifugation is carried out at 2°C to 8°C.
- [0035] The liquid is selected from the group consisting of: water, cell culture media, and a solution comprising a therapeutic agent.
- [0036] The collagen is type I collagen.
- [0037] The type I collagen is bovine collagen, human collagen, or recombinant human collagen.
- [0038] The crosslinking is carried out by a crosslinking agent selected from the group consisting of genipin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and UVA combined with riboflavin.
- [0039] In one other aspect, the invention encompasses a method of preparing a collagen composition, the method comprising:
- obtaining a collagen gel having a collagen concentration of 100 mg/ml to 190 mg/ml by centrifugation and ultrafiltration of a collagen solution, with the proviso that the collagen solution is substantially free of hydrochloric acid.
- [0040] In various aspects:
- [0041] The method further comprises: hydrating the collagen gel such that liquid content is increased 10-fold to 20-fold.
- [0042] The method further comprises: compressing the collagen gel.
- [0043] The method further comprises: neutralising the pH of the collagen gel.
- [0044] The concentration of the collagen is obtained by concentrating a collagen solution 10-fold to 50-fold by centrifugation in combination with ultrafiltration.

- [0045] The ultrafiltration is carried out with a molecular weight cut-off of 10 kDa.
- [0046] The centrifugation is carried out at 2000 x g to 4000 x g.
- [0047] The centrifugation is carried out at 2°C to 8°C.
- [0048] The collagen solution is 3 mg/ml to 10 mg/ml.
- [0049] The collagen solution comprises collagen provided in an acetic acid solution.
- [0050] The collagen is type I collagen.
- [0051] The type I collagen is bovine collagen, human collagen or recombinant human collagen.
- [0052] In yet one other aspect, the invention encompasses a collagen composition that comprises: a water content of 78% to 88% w/w, a transmittance of at least 0.85 for wavelengths of 570 nm, 750 nm, and/or 900 nm, a mean ultimate tensile strength of 1 MPa to 1.5 MPa at a strain rate of 0.5% s⁻¹, when uncrosslinked.
- [0053] In one further aspect, the invention encompasses a collagen composition prepared according to any one of the preceding aspects.
- [0054] In various aspects:
- [0055] The collagen composition comprises a water content of 78% to 88% w/w.
- [0056] The collagen composition comprises a transmittance of at least 0.85 for wavelengths of 570 nm, 750 nm, and/or 900 nm.
- [0057] The collagen composition comprises a mean ultimate tensile strength of 1 MPa to 1.5 MPa at a strain rate of 0.5% s⁻¹, when uncrosslinked.
- [0058] The collagen composition comprises a mean ultimate tensile strength of 1.5 MPa to 5.5 MPa at a strain rate of 0.5% s⁻¹, when crosslinked by a crosslinking agent selected from the group consisting of: genipin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and UVA combined with riboflavin.
- [0059] The collagen composition comprises an extracellular matrix component selected from the group consisting of: chitosan, proteoglycans, glycosaminoglycans, elastin, fibronectin, and laminin, and any combination thereof.
- [0060] The collagen composition comprises a growth factor selected from the group consisting of: FGF, VEGF, PDGF, EGF, IGF, TGF, HGF, KGF, and BMP growth factors, and any combination thereof.
- [0061] The collagen composition comprises a cell selected from the group consisting of: stem cells, progenitor cells, transient amplifying cells, mature cells, and any combination thereof.

[0062] The collagen composition comprises a cell selected from the group consisting of: keratocytes, chondrocytes, osteocytes, tenocytes, neurons, epithelial cells, keratinocytes, and any combination thereof.

[0063] The collagen composition comprises a protease inhibitor selected from the group consisting of: aprotinin, bestatin, pepstatin, tissue inhibitors of metalloproteinases (TIMP), and any combination thereof.

[0064] The collagen composition comprises a therapeutic agent selected from the group consisting of: anti-inflammatories, anti-microbials, antihistamines, steroids, sympathomimetics, beta receptor blockers, parasympathomimetics, parasympatholytics, prostaglandins, nutrients, vasoconstrictors, lubricants, anaesthetics, growth factors, and any combination thereof.

[0065] The collagen composition is prepared as a corneal tissue substitute, carrier for corneal cells, corneal implant, corneal inlay, corneal patch, or therapeutic contact lens.

[0066] In yet one further aspect, the invention encompasses a kit comprising the collagen composition of any one of the preceding aspects.

[0067] In still one further aspect, the invention encompasses a method of repairing or augmenting tissue of a subject, the method comprising: administering the collagen composition of any one of the preceding aspects to a tissue site of the subject, thereby providing repair or augmentation of the tissue.

[0068] In various aspects:

[0069] The cell is added following administration of the collagen composition.

[0070] The tissue is an epithelial tissue.

[0071] The tissue is skin.

[0072] The tissue comprises an epidermal, dermal, or hypodermal layer.

[0073] The tissue is corneal, retinal, conjunctival, or optic nerve tissue.

[0074] The tissue comprises a stromal layer.

[0075] The tissue is selected from the group consisting of: bone, muscle, nerve, and connective tissue, and any combination thereof.

[0076] The connective tissue is tendon or cartilage tissue.

[0077] The tissue is selected from the group consisting of: chondral, osteochondral, genitourinary, cardiovascular, neuronal tissue, and any combination thereof.

[0078] The tissue site is affected by one or more of: tissue thinning, tissue weakening, tissue loss, cell loss, matrix loss, collagen loss, or a defect in tissue form.

[0079] In even one further aspect, the invention encompasses a method of repairing or augmenting corneal tissue of a subject, the method comprising: administering the collagen composition of any one of the preceding aspect to a corneal tissue site of the subject, thereby providing repair or augmentation of the corneal tissue.

[0080] In various aspects:

[0081] The collagen composition comprises an extracellular matrix component selected from the group consisting of: chitosan, proteoglycans, glycosaminoglycans, elastin, fibronectin, and laminin, and any combination thereof.

[0082] The collagen composition comprises a growth factor selected from the group consisting of: EGF, KGF, HGF, IGF, and TGF growth factors, and any combination thereof.

[0083] The collagen composition comprises a cell selected from the group consisting of: stem cells, progenitor cells, transient amplifying cells, mature cells, and any combination thereof.

[0084] The collagen composition comprises a cell selected from the group consisting of: keratocytes, epithelial cells, and any combination thereof.

[0085] The cell is added following administration of the collagen composition.

[0086] The collagen composition comprises a protease inhibitor selected from the group consisting of: aprotinin, bestatin, pepstatin, tissue inhibitors of metalloproteinases (TIMP), and any combination thereof.

[0087] The collagen composition comprises a therapeutic agent selected from the group consisting of: anti-inflammatories, anti-microbials, antihistamines, steroids, sympathomimetics, beta receptor blockers, parasympathomimetics, parasympatholytics, prostaglandins, nutrients, vasoconstrictors, lubricants, anaesthetics, growth factors, and any combination thereof.

[0088] The collagen composition is prepared as a corneal tissue substitute, carrier for corneal cells, corneal implant, corneal inlay, corneal patch, or therapeutic contact lens.

[0089] The corneal tissue site is affected by one or more of the group consisting of: tissue thinning, tissue weakening, cell loss, tissue loss, matrix loss, collagen loss, and tissue irregularity.

[0090] The subject is affected by a condition selected from the group consisting of: corneal ectasias, corneal dystrophies, corneal ulcers, corneal infections, corneal wounds, and any combination thereof.

[0091] The corneal wound is caused by physical injury, chemical injury, radiation damage, and/or damage from particular medication.

[0092] The corneal wound is a surgical wound.

[0093] The foregoing brief summary broadly describes the features and technical advantages of certain embodiments of the present invention. Further technical advantages will be described in the detailed description of the invention and examples that follows.

[0094] Novel features that are believed to be characteristic of the invention will be better understood from the detailed description of the invention when considered in connection with any accompanying figures and examples. However, the figures and examples provided herein are intended to help illustrate the invention or assist with developing an understanding of the invention, and are not intended to limit the invention's scope.

BRIEF DESCRIPTION OF THE DRAWINGS

[0095] Figure 1: The scaffold has a high degree of transparency and uniformity of curvature on diffuse illumination (A) and on illumination with a slit-beam (B).

[0096] Figure 2: The mean $\pm$ standard deviation transparency of three samples before and after collagen crosslinking with riboflavin and UV-A. Collagen crosslinking resulted in decreased mean transmittance of UV light and increased transmittance of visible light.

[0097] Figure 3: Anterior segment OCT images of a normal human cornea (upper) and the scaffold (lower) show similar reflectivity profiles and thickness with maintenance of scaffold shape when removed from mould.

[0098] Figure 4: Degradation profile of uncrosslinked scaffolds, crosslinked scaffolds, and a corneal shield. Values shown are mean $\pm$ SD of three crosslinked scaffolds, three uncrosslinked scaffolds, and one corneal shield.

[0099] Figure 5: Light microscope images of concentrated collagen. (A) A cut edge of the collagen after concentration by centrifugation and drying is shown. Layers are clearly visible and the angle of alignment differs in the deeper layers compared to the surface. (B) A high degree of alignment is shown at higher magnification.

[00100] Figure 6: Scanning electron microscopy showing (A, B) a layered structure seen at the cross-section of two scaffolds and (C, D) the amorphous nature of the collagen on the surface of the scaffolds.

[00101] Figure 7: Scanning electron microscopy depicting closer view of layered structure seen in cross-section of scaffolding.

[00102] Figure 8: Light and confocal microscopy images showing porcine keratocyte infiltration into the scaffold (A, C) and human epithelial cells covering the surface of the scaffold (B, D). (A) Under light microscopy, porcine keratocytes covered the surface of the

scaffold (not shown) and infiltrated the depth of the scaffold in multiple layers after 17 days in culture. (B) Human epithelial cells fully covered the surface of a scaffold after 28 days. (C) Confocal microscopy of fluorescently labelled cross-sections of the scaffold demonstrate the presence of alpha SMA-positive and DAPI positive fibroblasts on the surface of the scaffold (circle), and keratocan-positive and DAPI-positive keratocytes (arrows) within the scaffold. Some non-specific background labelling from the keratocan antibody was visible on the surface of the scaffold, between the collagen layers of the scaffold, and on the keratocytes. (D) Confocal microscopy of fluorescently labelled cross-sections of the scaffold demonstrates the presence of cytokeratin-positive and DAPI positive epithelial cells (arrow) as a dense layer on the surface of the scaffold.

[00103] Figure 9: Confocal microscopy of fluorescently labelled cross-sections of the scaffold demonstrate the presence of alpha SMA-positive and DAPI positive (circle) fibroblasts on the surface of the scaffold, and keratocan-positive and DAPI-positive keratocytes (arrows) within the scaffold.

[00104] Figure 10: Confocal microscopy of fluorescently labelled keratocytes (Calcein-AM labelled) to show distribution of these cells throughout two different depths of the scaffold, (A) and (B). Cells were observed in a lamellar arrangement (C), and spread horizontally up to approximately 600 microns from the injection site over the course of 21 days, (A) and (B). Injection site circled.

[00105] Figure 11: Cross-section of mechanical device and measurement chamber for collagen scaffold.

[00106] Figure 12: Representative OCT image of collagen scaffold test strip inside the measurement chamber.

[00107] Figure 13: Representative cyclic-load curves taken from the various collagen scaffold samples for $0.1\% \cdot s^{-1}$.

[00108] Figure 14: Load response curve for the same samples when strained to failure at $0.5\% \cdot s^{-1}$.

[00109] Figure 15: Boxplot of Young's modulus associated with the various collagen scaffold samples, at a range of strain rates.

[00110] Figure 16: Boxplot of Young's modulus associated with the various collagen scaffold samples, at a 0.5% strain rate.

[00111] Figure 17: Boxplot of Young's modulus associated with the various collagen scaffold samples, at a 0.1% strain rate.

[00112] Figure 18: Boxplot of the ultimate tensile strength associated with the various collagen scaffold samples, at a 0.5% strain rate.

[00113] Figure 19: Cross-sectional area down length for an uncrosslinked sample of collagen scaffold.

[00114] Figure 20: Hydrated collagen scaffolds injected with keratocyte spheroids. (A) Representative image of post-injection scaffold under darkfield illumination. Arrows indicate the two injection sites containing keratocyte spheroids. (B) Representative cross-sectional view of a post-injection scaffold under brightfield illumination showing injected keratocyte spheroids. Note: (A) and (B) scaffolds were prepared separately. Scale bars: (A) 2000 μm (B) 200 μm .

[00115] Figure 21: Keratocyte migration from injection sites into hydrated collagen scaffolds. (A) 24 hours after injection. Cellular processes extend from the injection site into the scaffold (arrows). (B) 5 days after injection. Arrow indicates a keratocyte that has fully migrated into the scaffold. (C) 20 days after injection. Arrow indicates the keratocyte that has migrated furthest from the injection site. White line indicates the distance migrated. Note: (A) and (B) show different locations in the same scaffold, (C) scaffold was prepared separately. Dotted lines indicate the approximate edges of the injection sites. Scale bars: (A, B) 100 μm (C) 500 μm .

[00116] Figure 22: Confocal images of live keratocytes migrating through a hydrated collagen scaffold at 21 days post-injection. Keratocytes were loaded with calcein for fluorescence. (A) Maximum projection through an image stack. Dotted line indicates the approximate edge of the injection site. (B) Maximum projection of orthogonal (Z-X axis) sections taken through the original image stack, showing layered distribution of keratocytes. (C, D, E) Keratocytes at 9 μm (C), 131 μm (D), and 252 μm (E) from the top of the image stack demonstrating different patterns and distances of migration. Scale bars: 100 μm .

[00117] Figure 23: Migrating keratocytes in hydrated collagen scaffolds. (A) Bright field image of keratocytes 13 days post-injection. (B) Maximum projection through a confocal image stack of keratocytes loaded with calcein at 21 days post-injection. Arrowheads indicate keratocytes leading the migration and point in the direction of each keratocyte's migration. Dotted line indicates a column of keratocytes following a leading keratocyte. (A, B) Large arrows point away from the injection site and indicate the overall direction of migration. Scale bars: 50 μm .

[00118] Figure 24: Morphology and α -SMA expression in keratocytes in intact porcine cornea (A, B) vs. a hydrated collagen scaffold (C, D, E, F). (A, C) Representative bright field images showing quiescent morphology of keratocytes. (B, D, E, F) Representative maximum projections through confocal image stacks showing diffuse labelling of α -SMA in all keratocytes. Nuclei were labelled with DAPI. (C) 163 days and (D, E, F) 167 days post-injection, same scaffold. Keratocytes are located between the injection site and the migration front (C, D), at the migration front (E), and on the scaffold surface (F). Arrows indicate myofibroblastic cells with prominent stress fibres. Scale bars: 50 μ m.

[00119] Figure 25: Mean radius and thickness over time of scaffolds with and without injected keratocytes. Error bars indicate the standard error of the mean.

[00120] Figure 26: Transplantation of collagen scaffold into rabbit cornea for treatment following anterior lamellar keratoplasty. (A) Anterior lamellar keratoplasty in a rabbit model immediately post-operatively. (B) Anterior lamellar keratoplasty in a rabbit model 16 days post-operatively, viewed with diffuse white light. The corneal sutures were removed the day before. The grafted scaffold remains clear. (C) Anterior lamellar keratoplasty in a rabbit model 16 days post-operatively, with fluorescein instilled in the eye and viewed using a cobalt blue filter. The corneal sutures were removed the day before. The grafted scaffold is epithelialised.

[00121] Figure 27: Gene expression profile of human pre-macrophage cell line exposed to suture material or the collagen scaffold for 24 or 48 hours.

[00122] Figure 28: Biomechanical profile of tendons augmented with the collagen scaffold as compared to untreated tendons.

[00123] Figure 29: Histological scoring of repaired tendons augmented with the collagen scaffold as compared to untreated tendons.

DETAILED DESCRIPTION OF THE INVENTION

[00124] The following description sets forth numerous exemplary configurations, parameters, and the like. It should be recognised, however, that such description is not intended as a limitation on the scope of the present invention, but is instead provided as a description of exemplary embodiments.

Definitions

[00125] In each instance herein, in descriptions, aspects, embodiments, and examples of the present invention, the terms “comprising”, “including”, etc., are to be read expansively, without

limitation. Thus, unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like are to be construed in an inclusive sense as to opposed to an exclusive sense, that is to say in the sense of “including but not limited to”.

[00126] As used herein, “augmenting” refers to methods of increasing the bulk or strength of a tissue, including an epithelial tissue, or a muscle, nerve, or connective tissue. In relation to the eye, and in particular the cornea, “augmenting” means an increase in one or more of the thickness, hardness, elastic modulus, tensile strength, and regularity of the cornea, including the corneal tissue (e.g., the stromal layer). Methods of augmentation may be used to retain functionality to the tissue. Functionality for the eye includes transmittance, refraction, and sensing of light, and image perception. Augmentation methods may be performed in the presence or absence of a particular condition.

[00127] “Co-administration” or “co-administering” refers to the combined use of the collagen composition with one or more of: drugs or other therapeutic agents, biological agents, biological materials, or devices. This encompasses, as an example, the combined use of the collagen composition with one or more of such drug or other therapeutic agent, biological agent, biological material, or device for the eye. This includes the administration of co-formulations (i.e., integration of the composition with one or more of such drugs or other therapeutic agents, biological agents, or biological materials), as well as the simultaneous or sequential administration (i.e., use of the composition and one or more drugs or other therapeutic agents or devices as distinct formulations/materials). Similarly, “in conjunction” refers to the combined use of a therapeutic composition and a therapeutic device/procedure. This can include use of the composition preceding use of the device/procedure, simultaneously with the device/procedure, and/or following use of the device/procedure.

[00128] The collagen “composition” as described herein includes both crosslinked and uncrosslinked forms, and encompasses various scaffold forms, which may be moulded or unmoulded. The collagen composition may be formed as implants, inserts, prostheses, coatings, fillers, lenses, tubing, patches, sheets, dressings, etc., in accordance with the present disclosure.

[00129] A “condition” refers to a state of disease, defect, damage, injury, degeneration, or dysfunction relating to a tissue, or the cells associated with the tissue. Included are epithelial, muscle, nerve, and connective tissues. In relation to the eye, the condition may affect the corneal tissue (e.g., the stromal layer) or corneal cells (e.g., keratocytes), or other ocular tissues such as the tissues of the conjunctiva, retina, and optic nerve. Both acute and chronic conditions

are included. An acute eye condition may be, for example, a wound or ulceration. A chronic eye condition may be, for example, keratoconus or a corneal dystrophy.

[00130] The “cornea” as used herein refers to the transparent front part of the eye that covers the iris, pupil, and anterior chamber of the eye. It includes the corneal epithelium, Bowman’s layer, corneal stroma, Descemet’s membrane, and the corneal endothelium. Of particular interest is the stromal layer (also called the *substantia propria*) of the cornea, which comprises an extracellular matrix of regularly arranged collagen fibres along with keratocytes,

[00131] A “derivative”, as relating to a chemical derivative, refers to a compound that has been chemically modified. The present disclosure encompasses each of the chemical compounds described herein as well as any derivatives thereof, including chemically modified forms such as salts, hydrides, esters, and other modifications of the original compound.

[00132] “Isolated” as used herein, with particular reference to polypeptides, refers to a molecule that is separated from its natural environment. An isolated molecule may be obtained by any method or combination of methods as known and used in the art, including biochemical, recombinant, and synthetic techniques. To obtain isolated components, the polypeptides may be prepared by at least one purification or enrichment step. Of particular interest are polypeptides and peptides obtained by artificial means, i.e., non-natural, means. This includes but is not limited to, synthetic chemistry, recombinant technology, purification protocols, etc. Included are polypeptides isolated from natural, recombinant, or synthetic sources. Also included are polypeptides produced by chemical synthesis, or by plasmids, vectors, or other expression constructs that may be introduced into a cell or cell-free translation system. Such polypeptides are clearly distinguished from polypeptides as they naturally occur, without human intervention.

[00133] The terms “protein” or “polypeptide”, and other such terms, for simplicity, refer to the molecules described herein. Such terms are not meant to provide the complete characterisation of these molecules. Thus, a protein or polypeptide may be characterised herein as having a particular amino acid sequence, a particular 2-dimensional representation of the structure, but it is understood that the actual molecule claimed has other features, including 3-dimensional structure, mobility about certain bonds and other properties of the molecule as a whole. It is the molecules themselves and their properties as a whole that are encompassed by this disclosure. The terms “protein” and “polypeptide” are used interchangeably herein.

[00134] A collagen “polypeptide” refers to polypeptides obtained from any source, e.g., isolated naturally occurring polypeptides, recombinant polypeptides, and synthetic polypeptides, and to include polypeptides having the naturally occurring amino acid sequence

as well as polypeptides having variant amino acid sequences and fragment sequences, as described in detail herein.

[00135] Amino acid “sequence identity” refers to the amino acid to amino acid comparison of two or more polypeptides. A test sequence may be identical to a reference sequence (i.e., share 100% identity), or may include one or more amino acid substitutions. In preferred aspects, amino acid substitutions may possess similar chemical and/or physical properties such as charge or hydrophobicity, as compared to the reference amino acid. Sequence identity may be typically determined by sequence alignments at the regions of highest homology. Sequence alignment algorithms, for example BLAST® sequence alignment programs, are well known and widely used in the art. Based on the sequence alignment, the percent identity can be determined between the compared polypeptide sequences.

[00136] “Repair” of tissue means restoration following disease, defect, damage, injury, degeneration, or dysfunction of the tissue. Included are epithelial tissues, as well as muscle, nerve, and connective tissues. Repair may involve utilisation of a tissue substitute, prosthesis, implant, inlay, onlay, patch, and/or a scaffold of pre-grown cells, amongst other approaches. In relation to the cornea, “repair” refers to the restoration of one or more of the shape, thickness, regularity, clarity, continuity, hardness, elastic modulus, and tensile strength of the tissues eye, which may include that of the corneal tissue (e.g., the stromal layer). Methods of repair may be used to restore functionality to the tissue. Functionality for the eye includes transmittance, refraction, and sensing of light, and image perception. Repairs may be performed in the treatment of a particular condition, as described in detail herein.

[00137] A “scaffold” refers to a three-dimensional structure that can be used as a support for the repair and/or augmentation of tissue. Included are epithelial tissues, as well as muscle, nerve, and connective tissues. Specifically included are corneal tissues (e.g., the stromal layer) as described in detail herein.

[00138] The term “subject” refers to a human or non-human animal.

[00139] “Preventing” refers to stopping or delaying the onset of a medical condition. This includes an eye condition, or particularly a corneal condition, such as a disorder or other defect of the cornea. A preventative measure will result in the stoppage or delay of one or more symptoms of the condition, or a lessening of symptoms if such do arise. Prevention of a condition may involve augmenting tissue, as described in detail herein.

[00140] “Treating” refers to reducing, ameliorating, or resolving a medical condition. This includes an eye condition, or particularly a corneal condition, such as a disorder or other defect of the cornea. A treatment will result in the reduction, amelioration, or elimination of one or

more symptoms of the condition. Treatment of a condition may involve repair of tissue, as detailed herein. The compositions and methods of the invention may be used for treating various conditions, for preventing various conditions, or for both treating and preventing various conditions, as described in detail herein.

Collagen compositions and their preparation

[00141] The inventors have shown herein that a novel scaffold composed entirely of type I collagen can be produced to have high water content, high clarity, relatively slow degradation that can be prolonged by crosslinking, substantial mechanical strength, and to support the growth of epithelial cells and keratocytes. The scaffold shows notable efficacy in both *in vitro* and *in vivo* testing.

[00142] Type I collagen is the most dominant collagen type in the cornea (Birk et al., 1986), and its use for ophthalmic methods is preferred. The methods of the invention can also be used with other types of collagen, such as type II collagen, type III collagen, type IV collagen, up to and including type XXVIII collagen, or any combination of these collagen types, in any proportion. The collagen may originate from human, animal (non-human, e.g., from rat, bovine, aquatic organisms, etc), synthetic, or recombinant sources. Preferably, human collagen or recombinant human collagen is used for human treatment/prophylactic methods. Exemplary human collagen polypeptide sequences include NCBI sequence numbers P02452, P08123, P02458, P02461, P20908, P05997, P25940, P12107, P13942, Q7Z5L5, and Q8IZC6 for the human $\alpha 1(I)$, $\alpha 2(I)$, $\alpha 1(II)$, $\alpha 1(III)$, $\alpha 1(V)$, $\alpha 2(V)$, $\alpha 3(V)$, $\alpha 1(XI)$, $\alpha 2(XI)$, $\alpha 1(XXIV)$, and $\alpha 1(XXVII)$ chains, respectively, as well as variant amino acid sequences, and fragments thereof.

[00143] In specific circumstances, it may be desirable to use a collagen variant or fragment with the methods of the present invention. For example, the variant or fragment may exhibit at least 75% sequence identity to a polypeptide sequence noted above, preferably at least 80% identity, more preferably at least 85%, most preferably at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or about 100% sequence identity to an above noted sequence. It is of particular interest where the collagen variant or fragment exhibits biological activity, for example, activity that is similar or improved compared to the non-variant polypeptide.

[00144] The collagen can be used in any form, for example, atelocollagen, pro-collagen, or telocollagen forms, or any combination of these. Procollagen includes collagen molecules comprising either an N-terminal propeptide, a C-terminal propeptide or both. Telocollagen

includes collagen molecules lacking both the N- and C-terminal propeptides typically comprised in procollagen but still containing the telopeptides. The telopeptides of fibrillar collagen are the remnants of the N- and C-terminal propeptides following digestion with native N/C proteinases. Atelocollagen includes collagen molecules lacking both the N- and C-terminal propeptides comprised in procollagen and at least a portion of its telopeptides, but including a sufficient portion of its telopeptides such that under suitable conditions they are capable of forming fibrils.

[00145] Methods of isolating collagen from animals are known in the art. Dispersal and solubilisation of native animal collagen can be achieved using various proteolytic enzymes (such as porcine mucosal pepsin, bromelain, chymopapain, chymotrypsin, collagenase, ficin, papain, peptidase, proteinase A, proteinase K, trypsin, microbial proteases, and, similar enzymes or combinations of such enzymes) which disrupt the intermolecular bonds and remove the immunogenic non-helical telopeptides without affecting the basic, rigid triple-helical structure which imparts the desired characteristics of collagen. See e.g., US 3934852; US 3121049; US 3131130; US 3314861; US 3530037; US 3949073; US 4233360 and US 4488911 for general methods for preparing purified soluble collagen. The resulting soluble collagen can be subsequently purified by repeated precipitation at low pH and high ionic strength, followed by washing and resolubilisation at low pH.

[00146] In certain circumstances, it may be desirable to obtain fibroblasts from a subject, isolate collagen from these fibroblasts, prepare the collagen composition as described herein, and reinsert said collagen composition into the subject. Alternatively, it may be preferable to use recombinant sources of collagen. Recombinant collagen polypeptides, including any of the collagen forms, variants, and fragments thereof, may be expressed in any animal or non-animal cell, including but not limited to plant cells and other eukaryotic cells such as yeast and fungus. Plants expressing collagen chains and procollagen are known in the art. See, e.g., WO 2006/035442A3; Merle et al., 2002, FEBS Lett 515(1-3): 114-8; Ruggiero et al., 2000, FEBS Lett 469(1): 132-6; US 2002/098578 and US 2002/0142391, as well as US 6617431. It will be appreciated that genetically modified, variant forms of collagen are also encompassed, for example collagenase-resistant collagens and the like. See, e.g., Wu et al., 1990, Proc Natl Acad Sci 87: 5888-5892. In lieu of collagen, it is possible to substitute other polypeptides that exhibit lyotropic liquid crystalline behaviour. Non-limiting examples of other such polypeptides include silk proteins, cellulose, and polyglutamates.

[00147] Commercial collagen solutions are generally supplied at concentrations of 3 to 10 mg/ml. Commercial sources for collagen include VitroCol®, human collagen solution, type I

(Advanced BioMatrix, catalog #5007-A); OptiCol™ human collagen solution, type I (Cell Guidance Systems, catalog #M16S); human collagen solution, type I (Southern Biotech, catalog #1200-01S); recombinant human collagen, type I, alpha 1 chain (R&D Systems™, catalog #COL1A1); Collage™ recombinant human collagen, type I (Sigma-Aldrich®, catalog #C7624); human collagen solution, type II (Chondrex Inc., catalog #20052); and recombinant human collagen, type II, alpha 1 chain (R&D Systems™, catalog #COL2A1). Included as well is Cultrex® rat collagen, type 1 (Trevigen, catalog #3440-100-01). Medical grade collagen solutions can also be obtained from various sources, including Collagen Solutions (London, UK) and Symatase (Chaponost, France). In particular aspects, the collagen is provided in an acetic acid solution. For example, type I collagen may be provided in a solution of 0.1 M acetic acid, while type II and IV collagen may be provided in 0.25% acetic acid. In specific aspects, the collagen solution is provided as a monomeric solution.

[00148] In preferred aspects, the collagen used in the methods of the invention is not provided in a hydrochloric acid solution. It is also preferable that hydrochloric acid is not added in any substantial amount to the collagen during the methods of the invention. For example, the collagen solutions for use with the invention should contain less than 0.010 N HCl, or less than 0.005 N HCl, or less than 0.001 N HCl. Accordingly, the collagen is not solubilised in hydrochloric acid. Rather, the collagen is solubilised in acetic acid. The acidic solution containing the collagen may consist essentially of an acetic acid solution. The acidic solution containing the collagen may be substantially free of hydrochloric acid. The collagen solution may contain no hydrochloric acid. It has been found that commercially available collagen provided in solutions of hydrochloric acid yielded collagen gels having increased opacity and decreased tensile strength.

[00149] The collagen composition of the invention may be produced in accordance with the disclosed methods. As a first step, a collagen gel concentrate is obtained. The gel concentrate may be at least 70 mg/ml, at least 80 mg/ml, at least 90 mg/ml, at least 100 mg/ml, at least 110 mg/ml, at least 120 mg/ml, at least 130 mg/ml, at least 150 mg/ml, at least 160 mg/ml, at least 170 mg/ml, at least 180 mg/ml, at least 190 mg/ml, at least 200 mg/ml, at least 210 mg/ml, at least 220 mg/ml, at least 230 mg/ml, at least 240 mg/ml, or at least 250 mg/ml of a collagen polypeptide. The concentration may range, for example, from 50 mg/ml to 250 mg/ml; from 80 mg/ml to 230 mg/ml; or from 100 mg/ml to 190 mg/ml. In particular aspects, the concentration is about 125 mg/ml.

[00150] The gel concentrate may be obtained by centrifugal ultrafiltration of a collagen solution, although other methods may also be used. The starting collagen solution may be, for

example, 3 mg/ml to 10 mg/ml. The collagen of the starting solution may be provided in acetic acid as noted above. The collagen may be concentrated at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 40-fold, at least 50-fold, at least 60-fold, at least 70-fold, at least 80-fold, at least 90-fold, or at least 100-fold. The increase in concentration may range, for example, from 2-fold to 100-fold, from 5-fold to 60-fold, or from 10-fold to 50-fold.

[00151] Centrifugation may be carried out at, for example, at least 1800 x g, at least 2000 x g, at least 2200 x g, at least 2400 x g, at least 2450 x g, at least 2600 x g, at least 2800 x g, at least 3000 x g, at least 3200 x g, at least 3400 x g, at least 3600 x g, at least 3800 x g, at least 3890 x g, at least 4000 x g, at least 4200 x g, at least 4400 x g, at least 4600 x g, at least 4800 x g, at least 5000 x g, at least 5200 x g, at least 5400 x g, at least 5600 x g, at least 5800 x g, at least 6000 x g, at least 7000 x g, at least 8000 x g, or at least 9000 x g. The centrifugation may range, for example, from 1800 x g to 6000 x g; from 1800 x g to 4800 x g; or from 2000 x g to 4000 x g. In one aspect, the centrifugation is carried out at a range of 2400 x g to 2600 x g. In another aspect, the centrifugation is carried out at a rate of at least 4000 x g.

[00152] The centrifugation may be carried out, for example, at ambient temperature, or at about 25°C, at about 22°C, at about 20°C, at about 18°C, at about 16°C, at about 14°C, at about 12°C, at about 10°C, or at about 4°C. In particular aspects, refrigeration is used, such that the temperature ranges from 2°C to 8°C, or is kept at or about 4°C. For filtration, the molecular weight cut-off may be 100 kDa or less, 50 kDa or less, 20 kDa or less, 15 kDa or less, 12 kDa or less, 10 kDa or less, 8 kDa or less, 5 kDa or less, or 1 kDa or less. Centrifugation may be carried out for at least 10 hours, at least 15 hours, at least 20 hours, at least 25 hours, at least 30 hours, at least 35 hours, at least 40 hours, at least 45 hours, or at least 50 hours.

[00153] Filtration may be carried out by use of a centrifugation tube with an added filter. Such centrifuge tubes are commercially supplied, and readily available. Various membranes may be used for filtration, including but not limited to: polysulfone (PS) membranes, polyethersulfone (PES) membranes, cellulose acetate (CA) membranes, polyacrylonitrile (PAN) membranes, polyvinylidene fluoride (PVDF) membranes, polypropylene (PP) membranes, polyethylene (PE) membranes, and polyvinyl chloride (PVC) membranes. The size and shape of the gel concentrate may be tailored, for example, by addition of cylindrical prongs, or by use of a specialised centrifuge tube. For example, the dead stop region of the ultrafiltration centrifuge tube can be modified to direct the shape of the gel formed therein.

[00154] As a second step, the gel concentrate is hydrated. This can be done under centrifugal force. For hydration, the water content (or other liquid content) may be increased

in the gel concentrate by at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, at least 11-fold, at least 12-fold, at least 13-fold, at least 14-fold, at least 16-fold, at least 17-fold, at least 18-fold, at least 19-fold, at least 20-fold, or at least 21-fold. The increase in water content may range, for example, from 5-fold to 10-fold; from 10-fold to 20-fold; or from 15-fold to 20-fold. In a particular aspect, the water/liquid content is increased by about 20-fold. The invention is not limited to use of a particular liquid. Non-limiting examples include water, various buffers, cell culture media, preservative solutions, and solutions of therapeutic agents. Notably, a greater increase in liquid content produces greater transparency for the resulting gel. This is highly advantageous for ophthalmic applications, but may be deemed less important for other tissues. As such, the hydration step can be omitted or minimised for non-ophthalmic tissues. For example, the gel concentrate can be neutralised, moulded, set, and crosslinked (see below). Omission of the hydration step produces a gel with higher opacity that remains suitable for chondral tissues, and other tissues unrelated to the cornea, retina, etc.

[00155] Prior to hydration it may be desirable to compress the collagen gel. For example, a portion of the collagen gel can be pressed between two surfaces, e.g., between two glass slides or between a slide and a cover slip. The gel can be compressed to a range of 100 μm to 2 mm, e.g., 100 μm to 500 μm , or 500 μm to 1 mm, or 1 mm to 1.5 mm, or 1.5 mm to 2 mm. The compressed collagen gel can be removed from the glass slide. Hydration can then be carried on the compressed collagen gel. This can allow for more uniform hydration throughout the collagen gel.

[00156] As a third step, two or more aliquots of hydrated gel are combined together such that an aliquot of increased thickness is obtained. This may be done under centrifugal force. The centrifugation for the second and third steps may be carried out at the speeds and temperatures noted above. For the combination step, the filtration aspect noted above may be omitted. As before, the size and shape of the gel may be altered to suit by cylindrical prongs, specialised centrifuge tubes, or other means. This particular step can be repeated, as required, to obtain a gel with the desired thickness. Alternatively, the combining step (third step) and the earlier hydrating step (second step) can be omitted entirely, as described in detail herein.

[00157] As a further step, the collagen gel can be incubated under heat and humidity. This can be done to set the gel for further manipulation. The gel is considered to be set when it is firm and dry. A firm gel does not turn to a viscous liquid when heated. A gel is dry when it is substantially free of stickiness or tackiness to the touch. The temperature for this can be about 30°C, about 33°C, about 35°C, about 36°C, about 37°, about 38°C, about 39°C, or about 41°C,

or ranging from 30°C to 40°C, from 35°C to 39°C, or from 36°C to 38°C. In a particular aspect, the temperature is about 37°C. The humidity may be at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 98% relative humidity, or may range, for example, from 10% to 98%, from 30% to 98%, or from 50% to 98% relative humidity. In a specific aspect, the relative humidity is about 100%.

[00158] For the heating/humidifying step, it is possible to place a liquid, e.g., water, in the bottom of a plastic container, place the gel on a platform so that the gel remains above the liquid level, then seal the container and placed it in a heat chamber. During the heating/humidifying step, the collagen gel can be moulded or otherwise manipulated to form a particular shape or structure. For example, the gel can be placed into or onto a curved mould, and then pressed to form a curved shaped. The mould can be designed to allow a thicker area of scaffolding to form around the edges of the mould. This can assist with transplantation of the scaffold, e.g., by suturing or other types of fixation. Such shaping can also model the natural shape of the cornea, which is thicker around its perimeter. As one option, crosslinking may be carried out following the moulding step. Alternatively, crosslinking is carried out following a further dehydration-rehydration step, as described below.

[00159] The collagen gel can also be pH neutralised, as needed. Neutralisation may be particularly desirable where collagen has been prepared in acetic acid solutions. It may be expedient to carry out neutralisation during the heating/humidifying step noted above. Neutralisation may be achieved by incubation of the collagen gel with alkaline in liquid or vapour forms. For instance, solutions of sodium hydroxide, potassium hydroxide, calcium hydroxide, magnesium hydroxide, or other solutions may be applied. As non-limiting examples, the pH achieved may be about 7.0, about 7.5, about 8.0, about 8.5, or about 9.0, or may range, for example, from 7.0 to 9.0, from 6.5 to 8.5, or from 7.0 to 8.0. In a particular aspect, the pH may be about 8.

[00160] As still a further step, the gel set by heat and humidity may be dehydrated. Dehydration may be varied from less than 1% to 100% depending on the particular application required. For example, it may be desirable to achieve at least 1%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% dehydration, or from 20% to 100%, from 50% to 100%, or from 80% to 100% dehydration. In a specific aspect, complete dehydration is obtained. As yet one further step, the dehydrated gel can be rehydrated, and then crosslinked to provide further strengthening. Rehydration levels can be achieved at any of the levels noted above, including

an increase in liquid levels of 10-fold to 20-fold, as specifically noted above. In a particular aspect, the gel is maximally hydrated to equilibrium.

[00161] Crosslinking of the collagen composition may be achieved using one or more crosslinking agents, including but not limited to: genipin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), UVA alone or combined with riboflavin, diphenylphosphorylazide (DPPA), N-hydroxysuccinamide (NHS), glutaraldehyde (GA; Sheu et al., 2001; Barbani et al., 1995), polyepoxy, hexamethylene diisocyanate (HMDI), gamma-ray irradiation (Khor et al., 1997; Sung et al., 1996), nordihydroguaiaretic acid (NDGA; Koob et al., 2002a; Koob et al., 2002b; Koob et al., 2001a; and Koob et al., 2001b; Koob et al., 2003). Included also are crosslinking agents such as succinic anhydride, diisocyanates (e.g., hexamethylene diisocyanate (HMDIC)), activated and protected amino acids, a double activated polyethylene-glycol, or an activated carbon hydrate. Other crosslinking agents may be used in accordance with available methods.

[00162] An alternative or in addition to covalent bond crosslinking, it may be desirable to promote the formation of ionic bonds between collagen molecules. This can be achieved by polycationic molecules such as chitosan, which create ionic bonds between its numerous amine groups and the carboxyl groups of collagen. These bonds are strong enough to stabilise the biomaterial structure and form a strong mechanical strength (Shahabeddin et al., 1990; Berthod et al., 1994; Berthod et al., 1993). As a further option, enzymatic crosslinking agents like transglutaminase can be used to enhance tensile strength and enzymatic resistance of collagen-based biomaterial (Garcia et al., 2009; Yung et al., 2007; Khew et al., 2008).

[00163] The present invention is not limited to a particular crosslinking-bonding means. Any physical, chemical, or enzymatic crosslinking-bonding agent may be selected based on the tissue augmentation or repair to be achieved. It is expected that the agent(s) will confer mechanical strength and resistance to enzymatic degradation to the collagen composition, i.e., digestion by collagenase. The particular agent and the extent of crosslinking-bonding can be used to control the porosity and pore size of the material, and influence fibrous capsule thickness, blood vessel density, and the location of vessels within the three-dimensional porous scaffold (see, e.g., Joseph et al., 2004). Scaffolds with spacings/openings of greater than 60 μm may be prepared to allow deep penetration of capillaries and supporting extracellular matrix (see, e.g., Sharkawy et al., 1997). It is expected that spacings or openings of about 20 to 80 μm may be useful for the support of endothelial cells, such as for blood vessel formation, whereas openings greater than 100 μm may be useful for the support of osteoblasts, which are used for bone formation.

[00164] The collagen composition, as produced by disclosed method, may be tested to confirm its water content, transparency, and strength. The collagen composition may include about 70%, about 72%, about 74%, about 76%, about 78%, about 80%, about 82%, about 84%, about 85%, about 86%, about 88%, or about 90% water content, w/w; or from 75% to 85%, from 78% to 88%, or from 80% to 90% water content, w/w. In a particular aspect, the water content is about 85% w/w.

[00165] The collagen composition, prepared as a crosslinked or uncrosslinked gel, at an approximate thickness of 350 to 400 μm , may have a transmittance of at least 0.6, at least 0.65, at least 0.70, at least 0.75, at least 0.80, at least 0.85, or at least 0.90 for wavelengths of 570 nm, 750 nm, and/or 900 nm. In a particular aspect, the collagen composition has a transmittance of from 0.85 to 0.95 for wavelengths of 570 nm, 750 nm, and/or 900 nm. It will be understood that thinner samples of the collagen composition, e.g., gels having less than 350 μm thickness, will have higher levels of transmittance at the wavelengths tested.

[00166] The collagen composition, uncrosslinked, may have a mean Young's modulus at a strain rate of 0.5%/s from at least 2.5 MPa, at least 3.0 MPa, at least 3.5 MPa, at least 4.0 MPa, at least 4.5 MPa, at least 5.0 MPa, at least 5.5 MPa, at least 6.0 MPa, or at least 6.5 MPa, or may range, for example, from 2.5 MPa to 6.5 MPa, from 3.0 MPa to 6.0 MPa, or particularly, from 3.5 MPa to 5.5 MPa. The uncrosslinked composition may have a mean ultimate tensile strength at a strain rate of 0.5%/s from at least 0.8 MPa, at least 0.9 MPa, at least 1 MPa, at least 1.1 MPa, at least 1.2 MPa, at least 1.3 MPa, at least 1.4 MPa, at least 1.5 MPa, at least 1.6 MPa, at least 1.7 MPa, at least 1.8 MPa, at least 1.9 MPa, or at least 2 MPa, or may range, for example, from 0.8 MPa to 1.8 MPa, from 1 MPa to 2 MPa, or particularly, from 1 MPa to 1.5 MPa.

[00167] Upon crosslinking, the collagen composition may have a mean Young's modulus at a strain rate of 0.5%/s from at least 5 MPa, at least 7 MPa, at least 9 MPa, at least 11 MPa, at least 13 MPa, at least 15 MPa, at least 17 MPa, at least 19 MPa, at least 21 MPa, at least 23 MPa, at least 25 MPa, at least 30 MPa, at least 33 MPa, at least 35 MPa, at least 37 MPa, at least 39 MPa, at least 40 MPa, at least 41 MPa, at least 43 MPa, or at least 45 MPa, or may range, for example, from 5 MPa to 45 MPa, from 7 MPa to 37 MPa, or particularly, from 9.5 MPa to 35 MPa. The crosslinked composition may have a mean ultimate tensile strength at a strain rate of 0.5%/s from at least 1 MPa, at least 1.5 MPa, at least 2 MPa, at least 2.5 MPa, at least 3 MPa, at least 3.5 MPa, at least 4 MPa, at least 4.5 MPa, at least 5 MPa, at least 5.5 MPa, at least 6 MPa, at least 6.5 MPa, or at least 7 MPa, or may range, for example, from 1 MPa to 7 MPa, from 1 MPa to 6 MPa, or particularly, from 1.5 MPa to 5.5 MPa.

[00168] In certain aspects, it may be desirable to include one or more biological agents, therapeutic agents, or other matter within the three dimensional structure of the composition and/or on the surface of the composition. Various biomolecules may be included to enhance the mechanical strength and/or to modulate cellular functions such as migration, proliferation, and differentiation (see, e.g., Yeo et al., 1991; Teti, 1992; Huang-Lee et al., 1994; Zhong et al., 2009; Caissie et al., 2006; Antonicelli et al., 2009; Suh and Lee, 2002). Constituents of the extracellular matrix may be included, such as chitosan, proteoglycans, glycosaminoglycans, and various extracellular matrix proteins, e.g., elastin, fibronectin, and laminin. Cellular agents may also be included, for example, one or more protease inhibitors such as aprotinin, bestatin, pepstatin, and tissue inhibitors of metalloproteinases (TIMP), including TIMP-1, TIMP-2, TIMP-3, and/or TIMP-4, as well as one or more growth factors, recruiting factors, adhesion factors, and maturation factors. Non-limiting examples of adhesion factors include intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecule-1 (VCAM-1). Non-limiting examples of maturation factors include tumor necrosis factor- α , polyribocytidylic acid, lipopolysaccharide, CpG oligonucleotides, glia maturation factor-beta (GMF-beta).

[00169] The growth factor may be a member of the TGF (transforming growth factor) family, for example, any of TGF- β 1-3. The growth factor may be at least one of: bone morphogenetic proteins (BMP), including BMP-2, BMP-7, BMP-9, BMP-16; a vascular endothelial growth factor (VEGF); a platelet derived growth factor (PDGF); an insulin-like growth factor (IGF); an epidermal growth factor (EGF); a hepatocyte growth factor (HGF); a keratinocyte growth factor (KGF); a fibroblast growth factor (FGF); p15; or any other growth factor.

[00170] Various cells may also be included, for example, stem cells, progenitor cells, transient amplifying cells, mature cells, and any combination of these. Specific cell types associated with specific tissues may be utilised, including cells associated with epithelial, muscle, nerve, or connective tissues. Included are keratocytes, chondrocytes, osteocytes, tenocytes, neurons, epithelial cells, endothelial cells, fibroblasts, and keratinocytes, amongst others. In particular circumstances, cells may be added during and/or after the heating/humidifying step but before the noted dehydration step (see above). This may allow faster migration of cells into the collagen gel structure. In some circumstances it may be preferable to add cells after the noted dehydration step (see above). In other circumstances, it may be preferable to add cells after addition of the collagen composition to the target tissue. As one particular approach, it may be advantageous to obtain primary keratocytes from the

stroma of a subject, and then introduce such cells to the scaffold pre- or post-administration of the scaffold to the subject.

[00171] Various therapeutic agents may be included, for example, anti-inflammatory agents, cytostatic agents, anti-microbial agents, and/or particular ophthalmic drugs, amongst others. It may also be desirable to include one or more mineral components with the composition of the invention, for example, calcium phosphate such as tricalcium phosphate, ceramic materials, or biologically compatible metals.

[00172] In additional aspects, it may be useful to utilise mechanical cues (e.g. compression, stretching, or shear forces) or electrical cues along with the disclosed composition, such that implanted or native cells (e.g., chondrocytes, osteocytes, tenocytes, neurons, keratocytes, keratinocytes, epithelial cells) are assisted in repopulating a site of injury, defect, or disease. It may also be useful to provide surface modification or shaping of the composition, for example, using lasers such as excimer or femtosecond lasers, or other means. In addition, it may be desirable to provide internal modification of the composition, for example, to provide channels or pores within the scaffold structure, or to modify the shaping of the composition after implantation. Various lasers or other means may be employed for this.

Conditions affecting the eye and other tissues

[00173] The compositions described herein find particular use in repairing or augmenting bodily tissues, including tissues of the eye, as well other epithelial tissues, including skin tissues, which encompass epidermal, dermal, and hypodermal layers, along with muscle, nerve, and connective tissues. Connective tissues include tendon, ligament, cartilage, bone, and fat tissue. In specific aspects, the compositions may be used to repair or augment eye tissue, e.g., corneal, conjunctiva, retina, or optic nerve tissue. Thus, the compositions may be used to address ophthalmic thinning, weakening, cell loss, tissue loss, matrix loss, collagen loss, and/or irregularity. In this way, the compositions described herein may be utilised for various conditions affecting the eye, including conditions involving eye defects, disease, damage, injury, and/or degeneration.

[00174] In specific aspects, the invention encompasses methods for treating defects of the eye. In certain situations, the methods of the invention may also be used to prevent eye defects. The defects may be associated with a particular condition of the eye. Exemplary conditions include cicatricial pemphigoid, pterygium, as well as keratoconus, and related conditions, which include corneal ectasias such as keratoglobus, pellucid marginal degeneration, and

posterior keratoconus (see, e.g., Arffa, 1997; Krachmer et al., 1984; Rabonitz, 2004; Jinabhai et al., 2010). Congenital defects of the eye are also included. Amongst these are cornea plana and microcornea, the latter of which may be associated with foetal alcohol syndrome, Turner syndrome, Ehlers-Danlos syndrome, Weill-Marchesani syndrome, Waardenburg's syndrome, Nance-Horan syndrome, and Cornelia de Lange's syndrome. Included also is keratoglobus (mentioned above) that may be associated with Ehlers-Danlos syndrome type IV. Included as well are Leber's hereditary optic neuropathy and optic nerve hypoplasia.

[00175] In further aspects, the invention encompasses methods for treating damage or degeneration of the eye. In certain situations, the methods of the invention may be used to prevent eye damage. Damage or degeneration may be associated with a particular condition of the eye. Specifically included is corneal melt, for example, corneal melt associated with an inflammatory disorder, such as rheumatoid arthritis. Other exemplary conditions include scleritis, conjunctivitis, necrosis of the conjunctiva, conjunctival lesions, conjunctival inflammation, keratoconjunctivitis, optic neuritis, optic neuropathy, including ischemic optic neuropathy, and keratitis, such as marginal keratitis, stromal keratitis, exposure keratitis, neurotrophic keratitis, filamentary keratitis, rosacea keratitis, keratitis sicca, viral keratitis including herpes keratitis, fungal keratitis, protozoal keratitis, and other infectious keratitis, such as luetic interstitial keratitis, microsporidial keratitis, Thygeson's keratitis, and infectious crystalline keratopathy. Included also is ulcerative keratitis, also called peripheral ulcerative keratitis (PUK), which includes ulcerative keratitis that is associated with a systemic disease, such as rheumatoid arthritis Wegener's granulomatosis, systemic lupus erythematosus, relapsing polychondritis, and polyarteritis nodosa. Endophthalmitis is also included. Included as well are chronic corneal edema, Mooren's ulcer, dellen, phlyctenulosis, Terrien's degeneration, Salzmann's degeneration, spheroidal degeneration, and Fuch's dystrophy. Such conditions are well known and well characterised in the art. See, e.g., Jackson, 2008; Denniston, 2009; and Willshaw et al., 2000. Additionally included are stromal dystrophies, for example, lattice corneal dystrophy (e.g., type 1 and type 2), granular corneal dystrophy (e.g., type 1 and type 2), macular corneal dystrophy, Schnyder corneal dystrophy, congenital stromal corneal dystrophy, and fleck corneal dystrophy. Noted also is Stevens-Johnson syndrome. Additionally included is optic nerve atrophy, including atrophy caused by glaucoma, stroke, and other medical conditions. Included as well are retinal diseases, such as retinal dystrophies, e.g., retinitis pigmentosa, and conditions associated with aging, such as age related macular degeneration.

[00176] In still further aspects, the invention encompasses methods for treating injury to the eye. Included are injuries due to physical damage, chemical damage, radiation damage, and/or damage from particular medication. Injury to the eye may be associated with laceration, abrasion, erosion, puncture, membrane rupture, scarring, or ulcers, including melting ulcers, indolent ulcers, and superficial ulcers. Included also are injuries and other damage associated with eye surgery, including surgical wounds, corneal damage following radial keratectomy, and acute problems following keratoplasty, which include persistent epithelial defects. Specifically noted are rejected or failed corneal transplantations, and ophthalmic issues associated therewith. Additionally included are injuries associated with corneal melt, for example, corneal melt following surgery or other treatments of the eye (e.g., topical NSAID administration). Corneal melting may be attributable to infectious, inflammatory, or trophic causes. Conjunctival damage from topical treatments for glaucoma are further included. Included also are injuries and damage from toxic or caustic agents.

[00177] Conditions of the eye may be diagnosed by various methods, including fluorescein staining, which may include a Seidel's test, specular microscopy, corneal topography or tomography, pachymetry, ultrasound, slit lamps, corneal scrapes, and biopsies. Diagnosis may also involve assessments for visual acuity and/or opacification. Eye conditions may be associated with one or more symptoms of: pain, photophobia, foreign body sensation, reduced visual acuity, oedema, white cell infiltrate, fluorescein uptake, vascularisation, redness, and systemic symptoms such as headaches, nausea, and fatigue. Symptoms may also include blurry vision, double vision, haziness of vision, visual fatigue, foreign body sensation, problematic glare or halos, starburst patterns, ghost images, impaired night vision, squinting, excessive staring, excessive blinking, headaches, eye rubbing, eye strain, eye surface dessication, eye irritation, redness, and spasms of the eye.

[00178] The compositions also find use in repairing or augmenting other bodily tissues, including other epithelial tissues, as well as muscle, nerve, and connective tissues. In specific aspects, the compositions may be used to repair or augment such tissues. Accordingly, the compositions may be used to address tissue thinning, weakening, loss, cell loss, matrix loss, collagen loss, and/or defect in form. Thus, the compositions described herein may be utilised for various conditions affecting the body, including conditions involving tissue defects, disease, damage, injury, and/or degeneration. Specifically included are chondral and osteochondral conditions, as well as conditions affecting any tendon, ligament, muscle tissue, heart tissue, vascular tissue, genitourinary tissue, nervous tissue, tracheal tissue, or skin.

[00179] As non-limiting examples, cartilage defects and damage (e.g., knee or hip cartilage injuries or degenerative conditions) are encompassed, as well as defects and damage to the skin (e.g., wounds, burns, or surgical excision sites) vascular system (e.g., cardiovascular malfunction and venous or arterial pathologies), urogenital system (e.g., vesico-urethral reflux or incontinence), and nervous system (e.g., neuropathy, neural atrophy, or neuritis). In accordance with the present invention, augmentation may be carried out for cosmetic or therapeutic purposes. For example, tissue augmentation or reconstruction methods can be utilised in relation to facial tissue, breast tissue, pectoral tissue, calf tissue, tissue of the buttocks, or other bodily tissues. Bone and/or cartilage reconstruction may be necessitated by functional or aesthetic surgery.

Therapeutic compositions comprising collagen

[00180] As noted above, the compositions of the invention may be utilised for treating and/or preventing various medical conditions, including conditions affecting bodily tissues. Thus, the disclosed compositions may be used as a protective layer for tissue, tissue support, tissue substitute, and/or as a vehicle for delivery of cells, drugs, or other materials or agents to tissues. The compositions may be used for ocular tissues, including the cornea, conjunctiva, retina, and optic nerve. The compositions can be used also for cartilage, tendons, ligaments, muscles, bones, skin, heart, blood vessels, nervous tissues, genitourinary tract tissues, as well as other tissues.

[00181] In particular aspects, the compositions of the invention can be prepared for use as skin dressings, for example, for burn coverage applications or ulcer treatments (see, e.g., Yannas et al., 1982; Doillon and Silver, 1986; Peters et al., 1980). For skin coverings and skin replacements, the composition can be prepared to include one or more of: melanocytes, capillary-like networks, dendritic cells, sensory innervation, adipose tissue, and tissue reproducing psoriatic or sclerotic phenotypes (see, e.g., Caissie et al., 2006; Regnier, et al., 1997; Tremblay et al., 2005; Bechetoille et al., 2007; Blais et al., 2009; Trottier et al., 2008; Jean et al., 2009; Corriveau et al., 2009). An allogenic reconstructed skin can be constructed from the collagen composition that has been populated fibroblasts and overlaid by an epidermis. See, e.g., Apligraf®; Edmonds, 2009; Karr, 2008. In some circumstances, it may be desirable to use the compositions of the invention to direct mesenchymal stem cell delivery to the wound bed in collagen-based biomaterial for wound healing (see, e.g., Trottier et al., 2008; Nie et al., 2009; Altman et al., 2008a; Altman et al., 2008b). There are various methods for

preparing collagen materials as dermal substitutes and dressings, and the invention is not limited to any one strategy for preparation. See, e.g., commercial products such as Integra® (acellular collagen-GAG scaffold), Alloderm™ (human dermis), Terudermis® (collagen-silicone hybrid scaffold), Amniograph™ (amniotic membrane), and Oasis™ (porcine SIS). See also review by Parenteau-Bareil et al., 2010.

[00182] The compositions of the invention can also be prepared for use as eye tissue dressings, tissue supports, or tissue substitutes. For example, the compositions may be used as a corneal tissue substitute (e.g., in lieu of keratoprotheses), a carrier for corneal cells (e.g., sheets of limbal stem cells, epithelial or endothelial cells), a corneal implant, a corneal inlay, a corneal patch, or a therapeutic contact lens. For treatment of corneal conditions, it may be desirable to combine the collagen composition of the invention with stem cells. For example, the collagen composition of the invention can be used for the delivery of limbal epithelial stem cells to damaged cornea (see e.g., Levis and Daniels, 2009; Schwab, 1999; Zakaria et al., 2010; Shortt et al., 2009; Dravida et al., 2008; Grueterich et al., 2003). In certain circumstances, it may be desirable to utilise recombinant human collagen for the compositions of the invention, collagen produced by a subject's own fibroblasts, and/or surface modification of the collagen scaffold to modify endothelialisation (see, e.g., Dravida et al., 2008; Griffith et al., 2009; Liu et al., 2006; Lagali et al., 2008; Carrier et al., 2008; Rafat et al., 2009). See also Parenteau-Bareil et al., 2010. Alternatively, surface modification may be used to assist with cell guidance and/or to guide deposition of extracellular matrices. In the case of the cornea, surface modification may also be used to achieve various refractive benefits.

[00183] The compositions of the invention can also be prepared for use as dermal fillers. For example, the collagen compositions may be prepared as dermal implants or injectable formulations. Methods of adapting collagen compositions as injectable fillers are known in the art. See, e.g., commercially available and FDA approved dermal fillers such as Bovine Zyderm®, porcine Evolence™, human CosmoDerm® and Cymetra® (Bentkover et al., 2009; Mao et al., 2010). See also Gurney et al., 2007; Pons-Guiraud, 2008; Ellis and Segall, 2007. In particular aspects, the disclosed collagen compositions may be used to avoid adverse reactions that are associated with other dermal filler materials. See, e.g., Pons-Guiraud et al., 2010.

[00184] The compositions of the invention can further be prepared for use as delivery systems for tissues. For example, it may be desirable to use the compositions of the invention as delivery vehicles for drugs or other therapeutic agents (e.g., systemic or local acting drugs), or other materials. In particular aspects, the compositions may be used to deliver anti-inflammatory agents, antibiotics and/or other anti-microbial agents. As such, the compositions

of the invention may find particular use in the treatment of ulcers or for reconstructive surgery (see, e.g., Adhirajan, et al., 2009a, Adhirajan et al., 2009b, 36, 235–245; Sripriya et al., 2007; Shanmugasundaram et al., 2006; Sun et al., 2009; Liyanage et al., 2006; Bellows et al., 2008; Ansaloni et al., 2007). Controlled release systems may also be used in conjunction with the collagen compositions. Such systems may involve *in situ* gels, colloidal particles, nanoparticles, niosomes, liposomes, drug-loaded films (e.g., NOD®), and iontophoresis. In one particular aspect, hydrogel matrices may be integrated into or on the collagen composition or a covering comprised thereof. The compositions may be adapted as particular types of drug delivery systems, including ocular inserts and collagen shields. In various aspects, the collagen compositions may be used to deliver one or more of: cells, proteins, drugs or other therapeutic agents, and nucleic acids to a treatment or augmentation site (see, e.g., Glattauer et al., 2010; Takeshita et al., 2009; Takeshita et al., 2006; Sano et al., 2003). Cells for delivery include stem cells, progenitor cells, transient amplifying cells, and mature cells. The collagen compositions may be used, particularly, for gene therapy applications (see, e.g., Mulder et al., 2009). It is noted that the biodegradability of collagen and its low immunogenicity make it a substrate of choice for internal and topical pharmacogenomical applications. See, e.g., Parenteau-Bareil et al., 2010.

[00185] In other aspects, the compositions of the invention can be prepared for use as osteochondral tissue supports or tissue substitutes. For example, the disclosed compositions may be used as osteochondral implants. As to bone engineering, the collagen composition may be hardened, for example, by mineralisation with calcium phosphate (see, e.g., Harley et al., 2010; Du et al., 2000) and/or by crosslinking with other substances like hydroxyapatite or bushite (see, e.g., Ciardelli et al., 2009; Dubey and Tomar, 2009; Liao et al., 2009; Tamimi et al., 2008; Jayaraman and Subramanian, 2002). As to cartilage regeneration, the collagen composition may be provided in a flexible form. In osteochondral repairs, collagen type II may be preferable to other collagen types. However, autologous chondrocytes may be used with type I or II collagen structures, without any notable difference (see, e.g., Tebb et al., 2006; Glattauer et al., 2010). In some circumstances, it may be desirable to prepare sheet-like collagen scaffolds seeded with or without autologous cells (see, e.g., Gastel et al., 2001; Cook et al., 2006; De Franceschi et al., 2005; Freyria et al., 2009). Further, it may be advantageous to deliver differentiating mesenchymal stem cells directly via the collagen composition, to provide a permanent solution to osteochondral defects (see, e.g., Schneider et al., 2010). It may also be useful to optimise pore size and distribution for the collagen composition, to

maximise cell adhesion, proliferation, and migration (see, e.g., Murphy et al., 2010; see also Parenteau-Bareil et al., 2010).

[00186] The compositions of the invention can also be prepared for use as venous or cardiovascular tissue supports or tissue substitutes. The compositions may be used, for example, for heart valves and/or vascular conduits. The collagen compositions can be used to avoid the immunogenic and calcification problems associated with xenogenic heart valve matrices (see, e.g., Tedder et al., 2009; Eitan et al., 2010; Somers et al., 2009). Methods of adapting collagen material for use in cardiac and vascular reconstructive surgeries are known in the art (see, e.g., Teebken et al., 2009; L'Heureux et al., 1998; L'Heureux et al., 2001; Laflamme et al., 2005; McAllister et al., 2009; L'Heureux et al., 2007). See also commercially available products such as Cryolife® (Konuma et al., 2009; O'Brien et al., 1999; Bechtel et al., 2003). See also review by Parenteau-Bareil et al., 2010.

[00187] The compositions of the invention can also be prepared for use as tissue supports or tissue substitutes for the urogenital system. The collagen compositions can be used in lieu of enterocystoplasty and gastrocystoplasty techniques, which are beset by complications (see, e.g., Shekarriz et al., 2000; Mingin et al., 1999). Of particular interest are compositions useful for bladder augmentation or urethral stricture (see, e.g., Liu et al., 2009; Chen et al., 2009; Parshotam Kumar et al., 2010; Akbal et al., 2006; Fiala et al., 2007; Farahat et al., 2009; el-Kassaby et al., 2008). Acellular collagen compositions may be used, or alternatively, collagen compositions pre-populated with urothelial and/or muscle cells, or collagen produced by the subject's own fibroblasts (see, e.g., Bouhout et al., 2010; Atala et al., 2006; Magnan et al., 2006). Reviewed, e.g., by Parenteau-Bareil et al., 2010.

[00188] In additional aspects, the compositions of the invention can be prepared for use as neural tissue supports or tissue substitutes. For example, the collagen composition may be used as nerve conduits, which may be useful for peripheral nerve regeneration (see, e.g., Chamberlain et al., 1998; Archibald et al., 1991; Colin et al., 1984). It may be desirable to use acellular collagen compositions, which are moulded into a tubular shape (see, e.g., Hudson et al., 2004). Methods of adapting collagen materials for neural tubing are known in the art. Commercially available products include NeuraGen® from Integra™ amongst others. It may also be useful to employ the collagen composition in combination along with a particular pore orientation, neurotrophic factors, and/or cell delivery (see, e.g., Madaghiele et al., 2008; Bozkurt et al., 2007; Sun et al., 2009; Sun et al., 2007; Marchesi et al., 2007; Bozkur et al., 2009; Kemp et al., 2009). In other aspects, the collagen composition may be used to promote

axonal migration and myelination of neurons (Gringas et al., 2003; Gringas et al., 2008; Blais et al., 2009; see also Parenteau-Bareil et al., 2010).

[00189] The compositions of the invention can be used in combination with other implantable devices, for example, implantable stents, including cardiac, arterial, neuro (brain), urinary, and other stents, implantable power generators (IPGs), pacemakers, defibrillators, cardioverters, stimulators and/or lead systems for the brain, central nervous system (CNS) or peripheral nervous system, cardiac or other biological system, cardiac replacement valves, implantable sensors including glucose sensors, cardiac sensors, identity or tracking sensors (e.g., RFID), sensors to detect or measure oxygen, pH, temperature, ions, and the like, orthopedic implants, including tissue implants, such as facial implants for the chin, cheek, jawbone, and nose, implantable subcutaneous or percutaneous access ports, drain tubes such as Eustachian drain tubes, catheters such as urinary catheters, respiratory-assist tubes, and the like. The collagen composition or can be configured to substantially encase an implantable device or may cover only a portion thereof. The collagen compositions can be prepared as a three dimensional array of fibres or fibrils held together in or on the implantable device in any suitable manner including by their natural affinity to adhere together upon compression or extrusion, by using a sticky coating, such as a gelatinous coating, or any adhesive or glue, or by otherwise attaching the composition.

[00190] The composition and any coverings comprised thereof may also optionally comprise extruded, electrospun, braided and/or mesh collagen segments. This includes collagen components that are woven, interwoven, and/or interlocked in any manner, for example, three or more fibres or bundles of fibres together, including knitting and knotting and combinations of these or other constructions. The collagen composition can be provided as laminate fibres, foams, electrospun yarns or other formations. The composition may be used together with other scaffolds or scaffolding materials. Various scaffold construction methods may be used, for example, particulate leaching, porogen leaching, gas foaming, electrospinning, fibre mesh, fibre bonding, rapid prototyping (i.e., solid freeform fabrication), membrane lamination, and freeze drying.

[00191] In addition, the compositions of the invention may be used in conjunction with bioadhesive polymers or biological glues. Bioadhesive polymers may be macromolecular hydrocolloids with numerous hydrophilic functional groups, such as carboxyl-, hydroxyl-, amide, and sulphate capable of establishing electrostatic interactions. Exemplary agents include, at least, polyarylic acid (e.g., Carbopol®, carbophil, and polycarbophil) and carboxymethyl cellulose. Biological glues include transglutaminase tissue glues and various

fibrin sealants, for example, Artiss® or Tisseal® (Baxter, USA), Evicel™ (Johnson & Johnson, USA), Cryoseal® (ThermoGenesis, USA) Vitagel™ (Orthovita, USA), Tissucol™, Beriplast® (Aventis Behring), Crossseal™ (Omrix™ Biopharmaceuticals), and Quixil® (Johnson & Johnson Wound Management/Omrix™ Biopharmaceuticals).

Therapeutic methods and other methods using collagen compositions

[00192] As noted above, the compositions described herein find particular use in repairing or augmenting bodily tissues, including epithelial, muscle, nerve, and connective tissues. This includes, in particular, eye tissues such as the cornea (e.g., the stromal layer). In particular, the compositions may be used to provide enhanced or restored shaping, thickness, regularity, clarity, hardness, elastic modulus, tensile strength, or other functionality of the cornea. Thus, the compositions described herein may be used to address various conditions of the cornea and correct errors of the eye, and may be used as adjunct therapy with other eye treatments. The compositions may also be used to address conditions in other bodily tissues, such as tissue thinning, weakening, loss, cell loss, matrix loss, collagen loss, any other functional defects, and/or defect in form. In various aspect, the composition is used to enhance or restore the tissue functionality and/or appearance. Specifically encompassed are repairs/augmentations of other ocular tissues, including conjunctival, retinal, and optic nerve tissues, as well as chondral and osteochondral tissues, along with musculoskeletal, tracheal, vascular, cardiac, genitourinary, neuronal tissues, and skin.

[00193] The compositions described herein may be used in conjunction with various surgical procedures or other treatments. For example, the compositions can be used along with surgical and non-surgical methods used on the eye. Exemplary methods include but are not limited to: radial keratotomy (RK), including mini asymmetric radial keratotomy (MARK), hexagonal keratotomy (HK), photorefractive keratectomy (PRK), keratomileusis, laser *in situ* keratomileusis (LASIK), e.g., intraLASIK®, laser epithelial keratomileusis (LASEK), e.g., Epi-LASEK, automated lamellar keratoplasty (ALK), laser thermal keratoplasty (LTK), conductive keratoplasty (CK), limbal relaxing incisions (LRI), astigmatic keratotomy (AK), epikeratophakia, anterior ciliary sclerotomy (ACS), scleral reinforcement surgery, presbyopia reversal, and laser reversal of presbyopia (LRP). Also included are thermokeratoplasty, orthokeratology, enzyme orthokeratology, and chemical orthokeratology.

[00194] The compositions of the invention may be used in conjunction with surgical correction of non-refractive conditions, for example, surgical correction of a corneal tear. In particular aspects, the compositions described herein may be used in conjunction with specific

surgical methods performed on the cornea. Exemplary methods include but are not limited to: corneal transplant surgery, penetrating keratoplasty (PK), phototherapeutic keratectomy (PTK), pterygium excision, corneal tattooing, keratoprosthesis insertion (e.g., KPro or Dohlman-Doane), and osteo-odonto-keratoprosthesis insertion (OOKP).

[00195] The compositions of the invention may be used in conjunction with or in lieu of corneal collagen crosslinking. For example, the disclosed composition may be implanted into a subject after corneal crosslinking has been performed. Corneal crosslinking typically involves the use of riboflavin solution activated by exposure to UV-A light. Noted crosslinking methods include but are not limited to: corneal crosslinking with the epithelium removed (Dresden protocol, or epi-off), transepithelial crosslinking (epi-on), and accelerated crosslinking. Crosslinking procedures are generally available, and marketed as CXL, C3-R® CCL® and KXL® corneal crosslinking, amongst others. Corneal repair with the disclosed compositions can be used to treat subjects who have been deemed ineligible for crosslinking procedures, or as adjunctive therapy with corneal crosslinking, to slow or halt progressive corneal thinning.

[00196] The collagen compositions described herein may be co-administered with one or more drugs or other therapeutic agents. In various aspects, co-administration may be by simultaneous or subsequent administration with such agents, or by co-formulation with such agents. Depending on the condition being treated or prevented, the compositions described herein may be co-administered with one or more agents, which include but are not limited to: anti-inflammatories, antihistamines, steroids such as corticosteroids, sympathomimetics, beta receptor blockers, parasympathomimetics, parasympatholytics, prostaglandins, nutrients, vasoconstrictors, lubricants, anti-microbials, anaesthetics, and growth factors. Also included are biological agents or other biological material, e.g., various growth factors and cells as described above. Other agents or materials may be selected for inclusion with the composition; these may be chosen by the skilled artisan based on the condition and needs of the subject under treatment.

[00197] Exemplary anaesthetics include but are not limited to: benzocaine, bupivacaine, cocaine, etidocaine, lidocaine, mepivacaine, pramoxine, prilocalne, chloroprocaine, procaine, proparacaine, ropivacaine, and tetracaine. Exemplary anti-inflammatory agents include but are not limited to: aspirin, acetaminophen, indomethacin, sulfasalazine, olsalazine, sodium salicylate, choline magnesium trisalicylate, salsalate, diflunisal, salicylsalicylic acid, sulindac, etodolac, tolmetin, diclofenac, e.g., diclofenac sodium, ketorolac, ibuprofen, naproxen, flurbiprofen, e.g., flurbiprofen sodium, ketoprofen, fenoprofen, suprofen, oxaproxin, mefenamic acid, meclofenamic acid, oxicams, piroxicam, tenoxicam, pyrazolidinediones,

phenylbutazone, oxyphenthatrazone, pheniramine, antazoline, nabumetone, COX-2 inhibitors (e.g., celecoxib; rofecoxib; Celebrex®), apazone, nimesulide, and zileuton. Corticosteroids, including glucocorticoids, may also be used as anti-inflammatory agents. These include but are not limited to: hydrocortisone, dexamethasone, betamethasone, for example, betamethasone sodium phosphate, fluocortolone, fluoromethalone, lotoprendol, medrysone, prednisolone, prednisone, for example, methylprednisolone or methylprednisone, rimexolone, and lodoxamide.

[00198] Exemplary anti-microbial agents include but are not limited to: bacitracin zinc, chloramphenicol, chlorotetracycline, ciprofloxacin, erythromycin, gentamicin, norfloxacin, sulfacetamide, sulfisoxazole, polymyxin B, tetracycline, tobramycin, idoxuridine, trifluridine, vidarabine, acyclovir, foscarnet, ganciclovir, natamycin, amphotericin B, clotrimazole, econazole, fluconazole, ketoconazole, miconazole, flucytosine, clindamycin, pyrimethamine, folinic acid, sulfadiazine, and trimethoprim-sulfamethoxazole. Exemplary vasoconstrictors include but are not limited to: dipivefrin (Propine®), epinephrine, phenylephrine, apraclonidine, cocaine, hydroxyamphetamine, naphazoline, tetrahydrozoline, dapiprazole, betaxolol, carteolol, levobunolol, metipranolol, and timolol. Nutrients include vitamins, minerals, and other beneficial agents such as vitamin A, vitamin B₁, vitamin B₆, vitamin B₁₂, vitamin C (ascorbic acid), vitamin E, vitamin K, and zinc.

[00199] In specific aspects, the compositions of the invention are used to change the tissue structure of the cornea (e.g., the stromal layer) and in this way augment or repair the cornea. Other aspects of the anatomy, histology, and physiology of the cornea may also be affected by the collagen compositions. The collagen compositions may be hypertonic or hypotonic to induce changes in corneal hydration. For eye applications, the compositions may be co-administered with one or more eye drop formulations. These formulations may include but are not limited to: eye lubricating solutions, tear-replacing solutions, dry eye treatments, steroid and/or anti-microbial eye drops, glaucoma eye drops, allergy/anti-inflammatory eye drops, and conjunctivitis eye drops. The compositions may also be used in conjunction with other corneal inserts, corneal implants, or intrastromal rings, to assist in augmenting or repairing the subject's cornea. Included amongst corneal inserts are various corneal inlay devices. Examples of commercially available devices include intrastromal corneal ring segments, such as INTACS®, intrastromal corneal rings, such as KeraRings, scleral expansion bands (SEB), and Kamra™ inlays.

[00200] As the cornea is being augmented or repaired, various optometric measurements may be repeated to confirm that the treatment is progressing as planned and is adequate. Such

measurements may include assessment of visual acuity for near and far vision, keratometry measurements, corneal tomography, and objective and subjective retinoscopy. Modifications may be made to a treatment program based on these measurements. With each evaluation, a decision may be made whether to continue with the same treatment modality or whether a new treatment modality should be used. A decision can also be made with regard to the type collagen composition being used, e.g., inlay or onlay compositions, as noted below. Changes to the collagen composition or any co-administered agent or material can be made to induce the desired changes in the cornea over several weeks or several months. In certain aspects, weekly periodic revisions are performed during the first 8 weeks after treatment begins.

[00201] As described herein, the compositions of the invention may be prepared as corneal inlay or onlay compositions, for example, as full thickness or partial thickness corneal implants, corneal inlays, corneal patches, or therapeutic contact lenses. In various aspects, the corneal inlay or onlay composition may also act as a carrier and/or eluting device for one or more drugs or other therapeutic agents, biological agents, or biological materials. In other aspects, the inlay or onlay composition may be utilised with one or more drugs or other therapeutic agents or biological agents that are suitable for administration to the eye, e.g., eye drops, as described herein. In certain circumstances, it may be desirable to pre-administer the drug or other therapeutic agent or biological agent prior to placement of the inlay or onlay composition. The inlay or onlay composition may be unattached to the eye (e.g., as a therapeutic contact lens) or may be attached by any surgical or non-surgical means, for example, sutures or various adhesives/glues.

[00202] For corneal augmentation or treatment methods, computer software may be used to determine the inlay or onlay configuration that is most suitable for the subject and/or to determine the formulation and fit of the material. Utilisation of corneal inlay or onlay compositions may precede or follow, or may be used in conjunction with, eye surgery, e.g., refractive or transplant surgery. Medical assessment may be used to evaluate the subject (e.g., age, working needs of the subject, eye defect or disease, etc.), prescribing the use of inlay or onlay composition(s) to assist with the needed augmentation or repair of the eye. The inlay or onlay composition(s) that are prescribed and utilised by the subject are then able to support, augment, and/or stabilise the cornea, thereby inducing positive changes in the corneal tissue.

[00203] In certain aspects, the compositions of the invention may be used to prepare therapeutic contact lenses. The contact lens may be hard or rigid, or it may be a soft lens. Alternatively, the contact lens may comprise both hard and soft portions. If a soft contact lens is used, more positive or negative curvature can be induced in the cornea, and the discomfort in

the subject's eyes will diminish as he or she adapts to the contact lenses. If a hard contact lens is used, more mechanical pressure can be exerted on the cornea. The contact lenses are preferably designed to be gas permeable. For the calculation of the contact lenses the flattest keratometry is taken. One of skill in this art could also use the steeper keratometry or an average of both and based on this corneal curvature make the necessary calculations to flatten or steepen the radius of curvature of the anterior surface of the cornea. The peripheral base curve depends on the adaptation of the moulding contact lens and may be calculated to be 0.5 mm of radius greater than the central zone, but can vary depending on the design.

[00204] The diameter of the therapeutic contact lens used in accordance with the invention may be from 8.0 mm to 18.0 mm. In certain aspects, the therapeutic contact lens may be a hard contact lens with a diameter ranging from 8.0 mm to 12.0 mm. In other aspects, the therapeutic contact lens may be a soft contact lens with a diameter ranging from 13.0 mm to 15.0 mm. Soft contact lenses may cover the entire cornea and go from sclera to sclera. As previously noted, the therapeutic contact lens may be comprised of both hard and soft materials. The contact lens may be hard in the centre, out to approximately 12.0 mm, 13.0 mm, 14.0 mm, or 15.0 mm, and then soft in the periphery out to 16.0 mm, 17.0 mm, and 18.0 mm. A larger contact lens, preferably a soft contact lens, may be used at night as a therapeutic contact lens.

[00205] The compositions of the invention may be co-administered with one or more anaesthetics used to reduce the irritation to surrounding tissues. The compositions may be co-administered with one or more lubricants to improve the comfort of the subject during the treatment, particularly eye treatment. In other aspects, the composition may be co-administered with one or more anti-microbial agents, as described in detail herein. The composition may also be co-administered with one or more vasoconstrictors; this may be specifically advantageous for eye treatment. The person of skill in the art can determine the appropriate agents and materials for co-administration to the subject based on the condition being treated.

[00206] In certain aspects, the collagen composition may be provided as part of a kit. For eye applications, the kit may include one or more of: the collagen composition (e.g., prepared as an inlay or onlay composition), eye drops or other eye medication, and instructions for using the composition and eye medication. The composition provided with the kit may be formulated to include the collagen composition co-formulated with one or more drug or other therapeutic agent, biological agent, or biological material, or the kit may include the components as separate formulations, to be mixed together prior to administration, or to be administered together, i.e., by simultaneous or sequential administration. The container for the compositions of the invention may be clear, translucent, or opaque and may contain other properties or

combination of properties such as being glass lined, tamper proof, packaged in single or multiple aliquots, and any combination thereof.

[00207] The compositions of the invention also find use in various experimental applications. The collagen compositions may be used in both *in vivo* and *in vitro* research. For example, the compositions may be used to study cell behaviour such as migration and proliferation, as well as differentiation and phenotype expression. The disclosed compositions may be used to provide access to cellular membranes, for example, for electrophysiological protocols (see, e.g., Xu et al., 2009; Ma et al., 2004; O'Shaughnessy et al., 2003). The compositions may also be used as nervous system models to visualise motor neuron myelination (see, e.g., Gringas et al., 2008), or as disease models, for conditions such as osteoarthritis (see, e.g., Cortial et al., 2006). In other aspects, the compositions may be used to study the invasiveness of cancer cells, and the interactions between cancer cells and other cell types in a three dimensional environment (see, e.g., Che et al., 2006; Sabeh et al., 2009; Inoue et al., 2001). In addition, the compositions may be used to test anticancer drugs or other therapeutic agents (see, e.g., Shanmugasundaram et al., 2001). In still other aspects, the composition can be used to evaluate T cell migration patterns and their mechanisms (see, e.g., Stachowiak et al., 2008; Wolf et al., 2003). The compositions may be further used as anchoring systems for growing organs *ex vivo* (see, e.g., Spencer et al., 2008). See also review by Parenteau-Bareil et al., 2010.

EXAMPLES

[00208] The examples described herein are provided for the purpose of illustrating specific embodiments and aspects of the invention and are not intended to limit the invention in any way. Persons of ordinary skill can utilise the disclosures and teachings herein to produce other embodiments, aspects, and variations without undue experimentation. All such embodiments, aspects, and variations are considered to be part of this invention.

Example 1: Overview of experiments for synthesising and testing the collagen scaffold

[00209] The experiments described herein outline the development and characterisation of a collagen scaffold that is useful as a tissue substitute for human transplantation. Transparency, optical coherence tomography (OCT), and scanning electron microscopy (SEM) were used to analyse the structure of the scaffold. Water content was determined. The tensile strength was

assessed using a micro-mechanical analyser. *In vitro* biocompatibility was assessed by culturing the scaffold with epithelial or keratocyte spheres.

[00210] The mean scaffold transmittance was 0.72 at 358 nm, 0.91 at 750 nm, and 0.92 at 900 nm. OCT imaging confirmed that the scaffold maintained a corneal shape, with a central thickness of 502 μm and a reflectivity profile comparable to that of a normal human cornea. SEM of the scaffold revealed multiple lamellae on cross section. The mean water content was $88.7 \pm 0.7\%$ w/w. Young's Modulus at 0.5% strain rate for the uncrosslinked scaffold was 4.83 ± 1.39 MPa compared to 13.47 ± 5.73 MPa, 34.89 ± 22.14 MPa, and 12.32 ± 5.35 MPa for the scaffold cross linked with UVA + riboflavin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and genipin respectively (anterior and posterior human corneal stroma 9.72 ± 7.53 MPa and 2.04 ± 1.53 MPa respectively). Epithelial cells migrated over the scaffold to confluence. Keratocytes populated the scaffold and maintained a lamellar arrangement. These features are highly advantageous, and seen only for the inventors' composition.

Example 2: Synthesis of scaffold

[00211] Centrifugal ultrafiltration was performed by placing 10 mL of 5 mg/mL Cultrex® 3D culture matrix rat collagen I (Trevigen, Gaithersburg, Maryland, USA) in a Vivaspin® Turbo 15 unit (Sartorius AG, Goettingen, Germany) with a 10 kDa molecular weight cut off (MWCO) polyethersulfone (PES) membrane. The solution was spun in a centrifuge unit (Sigma 3K15) at 4°C at 2500 rpm for 30 hours, thereby increasing the concentration of the solution to 125 mg/ml (volume 0.4 ml).

[00212] The resulting firm collagen gel was divided into eight pieces of equal weight. Each piece was placed in a separate standard centrifuge tube and spun at 4 °C at 2500 rpm for 10 hours in 50 mL Milli-Q water to hydrate the collagen. Two pieces were then combined by centrifuging in a single centrifuge tube in 50 mL Milli-Q water for an additional hour at 4 °C at 2500 rpm. The combined hydrated gel was then removed and placed in a mould. Two types of moulds were used to produce either flat or cornea shaped scaffolds. The flat mould consisted of a plastic ring (internal diameter of 12 mm and a height of 8 mm) placed on a glass slide with a glass cover slip placed on top of the mould. The cornea shaped mould consisted of two rigid contact lenses spaced 8 mm apart.

[00213] After the collagen gel was placed in the respective mould, 8 μL of 1 M NaOH was applied to the surface of the gel. The mould was then placed in a humid chamber at 37°C for 4 hours. The plastic ring was removed, and the resulting cylindrical gel (placed on a glass slide

and covered by a glass coverslip) was placed in a dry incubator at 37°C for at least 12 hours to allow complete dehydration. The dehydrated gel was subsequently rehydrated by immersion in Milli-Q water for 4 hours. This allowed for maximal hydration of the gel to equilibrium.

Example 3: Crosslinking of scaffold

[00214] Dehydrated scaffolds were soaked in 0.1 % riboflavin in phosphate buffered saline. Light irradiation was then commenced using an ultraviolet A (UVA) double diode 370 nm light source located approximately 10 mm above the scaffold. This produced a radiant energy of 3 mW/cm² or 5.4 J/cm². Irradiation was performed for 30 minutes, then the sample was turned over and the irradiation repeated for a further 30 minutes. The sample was then flushed and soaked in 800 ml of Milli-Q water for 24 hours to remove the riboflavin from the scaffold.

Example 4: Transmittance of scaffold

[00215] Each flat scaffold sample was cut into a 6.5 mm diameter circular button using a corneal trephine and placed in a well in a 96 well plate with 200 µL MilliQ water. The absorbance of three samples to light at 358, 420, 570, 750 and 900 nm was measured using a microplate spectrophotometer (BioTek® Synergy HT Multi-Mode Microplate Reader, BioTek Instruments Inc., Winooski, VT, USA), with the absorbance of Milli-Q water control subtracted from each reading. The transmittance of light through the sample at a particular wavelength was calculated as $10^{(-\text{absorbance})}$. The transmittance of the three samples before and after collagen crosslinking was compared.

Example 5: Water content of scaffold

[00216] Three scaffolds were dried at room temperature for at least 12 hours and the dry weight measured. The dehydrated samples were subsequently hydrated to equilibrium by soaking in Milli-Q water for 4 hours and the wet weight measured. The following formula was used to calculate the percentage water content of the hydrated scaffolds:

[00217] Water content = $\frac{(\text{wet weight} - \text{dry weight})}{\text{wet weight}} \times 100$

Example 6: Degradation of scaffold *in vitro*

[00218] The degradation profile of scaffolds before and after crosslinking was assessed using a collagenase degradation assay. A 72 hour Soft Shield® Corneal shield (Oasis®, San Dimas, CA, USA) with a weight of 45 mg was also included for comparison. Samples were individually weighed and soaked in PBS with saturated CaCl₂ for 3 days to allow for swelling before degradation was commenced. One unit collagenase (collagenase from *Clostridium histolyticum*, type 1A, Sigma C9891) in 0.5 Unit/mL PBS with saturated CaCl₂ was applied per 50 mg wet weight scaffold. The scaffolds were then weighed five times a week for two weeks. The results were expressed as percentage weight remaining.

Example 7: Light microscopy analysis of concentrated gel

[00219] Light microscopy was performed to analyse the macro-structure of the collagen gel immediately after centrifugation. The concentrated collagen gel (final volume 0.4 mL) was removed from the Vivaspin® tube and dehydrated at 37°C for 24 hours in a dry incubator then viewed using light microscopy.

Example 8: Scanning electron microscopy of scaffold

[00220] Scanning electron microscopy was performed to analyse the microstructure of the scaffold. A scaffold was fixed in 4% paraformaldehyde for 20 minutes, washed three times for 15 minutes in PBS, then transferred through 15 minute washes of water, 30%, 50%, 70%, 90% and 100% ethanol. The sample was cut in half with a tissue chopper (Starrett 263M, USA) for cross-sectional and *en-face* views. The samples were critical point dried and platinum coated one hour before images were taken with a scanning electron microscope (Philips FEI XL30 S-FEG).

Example 9: Stress, strain, and stiffness measurements for scaffold

[00221] The normal pressure in the eye ranges from 1 kPa to 2.7 kPa (8 mmHg to 21 mmHg). The equivalent wall stress, computed by treating the eye as a spherical, thin walled pressure vessel, ranges from 6.4 kPa to 16.8 kPa. During rubbing, the wall stress in the eye can exceed 74 kPa (555 mm Hg). Therefore, the mechanical properties of the collagen scaffolds are important in determining their suitability for implantation.

[00222] Mechanical properties such as stiffness and ultimate tensile strength can be modified by crosslinking the collagen scaffolds with various reagents. The goal of these experiments was to compare the ultimate tensile strength, and tensile stiffness of engineered collagen scaffolds against representative corneal donor tissue, and further to assess the effect of available crosslinking reagents on these same properties. For these experiments, the 3-D geometry of the collagen scaffold samples were directly imaged using optical coherence tomography (OCT), concurrently with high powered transmission microscopy. The length and position of the sample was controlled within the field of view using dual voice-coil motors. These capabilities combined allowed accurate quantification of stress throughout the entire volume of the collagen scaffold samples.

[00223] To prepare the samples, planar, circular portions of engineered collagen were bisected and one of three potential treatments was applied to crosslink each half sample. The three reagents used were genipin, EDC, or riboflavin with application of UV light. Genipin is a natural crosslinker for collagen, gelatin, and chitosan. It is derived from *Gardenia jasminoides* and has been shown to have a dose dependent effect on porcine corneal stiffness (Avila and Navia 2010). EDC is 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide. It has been used extensively for crosslinking collagen scaffolds, and for attachment of other molecules to collagen scaffolds (Pieper, Hafmans et al. 2000; Everaerts, Torrianni et al. 2008; Rafat, Li et al. 2008). Riboflavin is a photosensitiser which serves to crosslink collagen matrices when introduced in conjunction with ultraviolet light. It has been used in the past to treat keratoconus (Wollensak 2006; Wollensak and Iomdina 2009).

[00224] Twelve scaffold samples were bisected and one half of each sample was crosslinked using either UV and riboflavin (n=2), genipin (n=2), or 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) (n=2). The remaining half of each sample remained uncrosslinked (n=6). Tensile strength of the scaffold samples was assessed. For comparison, a human donor cornea was also assessed. Rectangular strips (~100 μ m x 300 μ m x 3 mm) were cut from each half sample in the same orientation as the original bisecting cut. Each strip was hydrated in Millipore distilled water for a period of no less than 3 hours before being transferred into the mechanical tester. In parallel, a corneal button was obtained from the Ophthalmology donor bank after fully informed consent from the New Zealand National Eye Bank. All human tissue was used with approval from the Northern X Regional Human Ethics Committee. The corneal epithelium, endothelium, and Descemet's membrane were removed from the button and the stroma was further dissected into anterior and posterior stroma layers at the midpoint of the thickness. Each layer of the stroma was bisected and then sectioned in similar fashion to

the engineered scaffolds. The sections were kept in organ culture medium before they were transferred to the measurement chamber, upon which time they were hydrated in Millipore water.

[00225] To mount the samples, individual specimen strips were attached end-on between two glass capillaries using cyanoacrylate. At one end, the capillary was attached to a linear motor. At the other, it was attached to a cantilever force transducer. The force transducer rode on a second linear motor. The two motors acted in concert to allow the translation of the specimen from a hydrated mounting chamber into a 1 mm square i.d. capillary which forms the measurement chamber of the apparatus. Distilled water was passed continuously through the measurement chamber to hydrate the sample.

[00226] To quantify force, the deflection of the stainless steel cantilever was measured using a heterodyne laser interferometer with a resolution of 3.1×10^{-10} m and a measurement bandwidth of 20 MHz down-sampled to 500 kHz. The apparent stiffness of the cantilever, for the laser configuration used in these experiments, was calibrated at 1400 N/m using a strain-gauge force transducer (FUTEK LSB200). The first resonant frequency of the unloaded cantilever and collagen mounting system was measured at approximately 1120 Hz and the noise-equivalent force, as quantified by the standard deviation of force noise over a measurement bandwidth of 1 kHz, was 2.23 μ N. In addition to measuring the cantilever deflection, the heterodyne interferometer was also used to measure the displacement of the two linear actuators attached to either end of the sample, one of which carried the force transducer.

[00227] It is considered best practise to map measurements of force and displacement to stress and strain, using the cross-sectional area of the specimen and its initial length respectively. Doing so allows comparison of stiffness estimates between samples. This is easy to perform with the types of materials typically tested for engineering applications, such as metal alloys and ceramics. With biological materials the measurements of cross-sectional area are much more difficult. Compliance in the tissue makes compression measurement devices inappropriate, and typical microscope based measurements offer poor ability to resolve the three-dimensional shape. These considerations were taken into account when assessing collagen scaffold specimen geometry.

[00228] To address issues of force and displacement mapping, an optical coherence tomograph (OCT) was integrated into the mechanical tester. An OCT takes advantage of differences in refractive indices of samples to generate an intensity profile through the thickness of tissue. Utilising the OCT, a laser beam was passed through the sample under study and reflected light was interfered with a reference beam to generate the profile. The interference

pattern was picked up by a line scan camera and converted to a structural depth map using an inverse Fourier transform. For a target immersed in water within the capillary, the OCT showed spatial resolution $10\ \mu\text{m}$ (height) $\times$ $10\ \mu\text{m}$ (width) $\times$ $2.7\ \mu\text{m}$ (depth) (Cheuk, Lippok et al. 2014).

[00229] Depth profiles from the OCT were combined vertically to form “B-scan” images and then the images are stacked to create a pseudo volume. An estimation of the cross-sectional area down the length was obtained in post-processing. The Weka suite of classifier algorithms implemented in the image processing software ImageJ (Ignacio Arganda-Carreras 2015) was used to segment the collagen cross-sections from the background of each image. An estimate of cross-sectional area was then computed and the mean for each acquired volume (representing alternating load and unload steps) was estimated. Cyanoacrylate at either end of the sample was discernible from collagen by its apparent intensity in the image. Sections which contain cyanoacrylate were not included in the estimate of mean cross-sectional area for the sample volume.

[00230] The mechanical testing protocol was carried out as follows. The length of each sample was increased manually until a stress equivalent to the *in vivo*, resting wall-stress (12 kPa) was observed after a settling period. The stress was computed using an estimate of minimum cross-sectional area gathered from an initial scan using the OCT. Once the target stress had been reached, the sample was scanned again using the OCT and the stress recomputed with a revised estimate of average cross-sectional area under load. If this modification caused the apparent stress to exceed the maximum *in vivo* range, the “resting” strain was reduced. In practise this precaution proved unnecessary.

[00231] The sample was then taken through a series of cyclic loading experiments, first holding strain-rate constant at $0.01\ \%\cdot\text{s}^{-1}$ and varying strain. Subsequently holding target strain constant at 1 % and varying strain-rate. A representative full-volume scan was acquired using the OCT during the load and unload step the cyclic loading pattern at a combination of 1 % target strain and $0.1\ \%\cdot\text{s}^{-1}$ strain-rate. Finally the samples were strained to failure at a rate of $0.5\ \%\cdot\text{s}^{-1}$.

Example 10: Cellularisation of the scaffold

[00232] To investigate whether the scaffold supports corneal keratocyte growth, porcine keratocyte spheres were cultured and seeded onto scaffolds.

[00233] Whole porcine eyes were sterilised by 4% povidine iodine and 4% sodium thiosulfate. Corneal epithelial cells were removed by scraping using a keratome before and after immersion for 40 minutes at 37°C in 1.2 U/mL of dispase (Gibco® Life Technologies, USA) in MEM (Gibco®, Life Technologies, USA). The de-epithelialised cornea was incubated at 37°C for 20 to 24 hours in 1 mL of MEM (Gibco® Life Technologies, USA) containing 2 mg/ml collagenase (Sigma-Aldrich®, USA) and 0.5 mg/mL hyaluronidase (Sigma-Aldrich, USA).

[00234] A single cell suspension was obtained and cultured in Advanced DMEM (Gibco®, USA) supplemented with 1% GlutaMAX™ (Gibco®, USA), 1% antibiotic/antimycotic (Gibco®, USA), 2 ng/mL recombinant human EGF (Invitrogen, USA), and 1 ng/mL recombinant human bFGF (Invitrogen™, USA). Cells were cultured on a 22 mm round glass coverslip in a well of a 6-well cell culture tray. Keratocytes cultured in this way were known to aggregate and form spheres (Funderburgh et al., 2008).

[00235] After 10 days in culture, keratocyte holoclones were pipetted onto each uncrosslinked scaffold in individual wells of a 6-well cell culture tray. Scaffolds were incubated overnight to allow the holoclones to settle down onto the scaffold surfaces. Remaining medium was removed and replaced with Advanced DMEM (Gibco®, USA) supplemented with 1% GlutaMAX™ (Gibco®, USA), 1% antibiotic/antimycotic (Gibco®, USA) and 25 ng/mL recombinant human IGF-II (Gibco®, USA). Medium was replaced twice weekly and keratocytes on scaffolds monitored using a Leica DMIL IL LED inverted microscope (Leica Microsystems, Germany). Digital images were acquired with a Leica MC120 HD microscope camera (Leica Microsystems, Germany), controlled by a PC computer running Leica Application Suite version 4.4.0 (Leica Microsystems, Germany).

[00236] After 42 days, scaffolds were fixed in 4% paraformaldehyde in PBS for 19 hours at 4°C, washed in PBS, and cryoprotected by 20% sucrose for 4 hours at room temperature and 30% sucrose for 24 hours at 4°C. Scaffolds were embedded in Tissue-Tek Compound (Sakura, Japan), frozen in liquid nitrogen, and stored at -20°C until cryosectioning. Fixed frozen scaffolds were cut into 16 µm thick sections with a Microm HM550 cryostat (Thermo Fisher Scientific, Germany) and stored on glass slides at -20°C prior to immunohistochemical labelling.

[00237] Slides were labelled with rabbit anti-keratocan primary antibody (Santa Cruz, Dallas, Texas, sc-66941, 1:100), as a marker for keratocytes, and mouse anti-alpha smooth muscle actin primary antibody (α -SMA, Novocastra, NCL-SMA, 1:50), as a marker for myofibroblasts, followed by goat anti-rabbit Alexa568 secondary antibody (Life Technologies,

USA, A11011, 1:400) and goat anti-mouse Alexa488 secondary antibody (Life technologies, USA, A10680, 1:400). Slides were mounted with ProLong® Gold Antifade Mountant with DAPI (Life technologies, USA, P1014). Images were taken with an Olympus® FV1000 confocal laser scanning microscope (Olympus®, Japan).

[00238] To investigate whether the scaffold supports corneal epithelial cell growth, a corneal limbal rim from a 74 year old human donor with prior research consent was obtained from the NZ eye bank post-surgery. A 3 mm by 3 mm by 200 µm piece of limbus was dissected and placed epithelial-side down onto the flat scaffold. The scaffold and the epithelial explant were cultured in advanced DMEM supplemented with 1% GlutaMAX™ and 1% antibiotic/antimycotic, with medium changed twice a week. After 28 days, light microscope images were taken. The scaffold was fixed and cryosectioned as described. Slides were incubated with mouse anti-cytokeratin antibody (DAKO M3515, 1:50) for epithelial cells followed by goat anti-mouse Alexa568 antibody (Life Technologies A-11004, 1:400). Slides were mounted with ProLong® Gold Antifade Mountant with DAPI. Images were taken with an Olympus® FV1000 confocal laser scanning microscope.

[00239] In additional experiments, a collagen scaffold was seeded with 10 day old porcine keratocyte spheres, which were prepared as described above. The scaffold was seeded after the setting step of Example 2; that is, after the neutralising/heating/humidifying step, but before the last noted dehydration then rehydration steps. This was done to expedite cell growth into the scaffold structure. Spheres were injected vertically the scaffold and cultured for 3 weeks. To carry out injection, a vertical channel was formed in the gel using a 20 gauge micro vitreoretinal (MVR) blade. Porcine keratocyte spheres were placed in the channel using a 200 µL pipette. The spheres ranged in size from approx. 50-350 µm in diameter. Culturing was carried out in Advanced DMEM (Gibco®, USA) supplemented with 1% GlutaMAX™ (Gibco®, USA), 1% antibiotic/antimycotic (Gibco®, USA) and 25 ng/mL recombinant human IGF-II (Gibco®, USA). Medium was replaced twice weekly and keratocytes on scaffolds monitored using a Leica DMIL IL LED inverted microscope (Leica Microsystems, Germany). The seeded scaffold was incubated in 2 µM Calcein-AM (Life Technologies) for 1 hour, then imaged on a ZEISS 710 LSM confocal microscope using a 10 X objective lens.

Example 11: Statistical analysis

[00240] Transparency and water content before and after crosslinking were compared using paired Student's t-tests. Degradation and tensile strength of uncrosslinked scaffolds compared

to crosslinked scaffolds were compared using unpaired Student's t-tests. A p -value less than 0.05 denotes statistical significance. Tensile strength data were subject to histogram analysis for normality, and the data were found to be not normally distributed, therefore a log transform was applied. The transformed data was examined for the effect of cross-linking, strain-rate, and strain on apparent stiffness using analysis of variance, and the effect of cross-linking between at least one pair of cross-linking treatments was found to be highly significant ($p < 0.0001$).

Example 12: Results: scaffold transparency, thickness and shape, and water content

[00241] The scaffold was transparent and uniform upon diffuse illumination and illumination with a slit-beam (Figure 1). The scaffolds consistently showed a high degree of transparency at visible wavelengths. The mean scaffold transmittance was 0.72 ± 0.04 (mean $\pm$ standard deviation (SD), average of 3 scaffolds) at 358 nm, 0.81 ± 0.03 at 420 nm, 0.88 ± 0.02 at 570 nm, 0.91 ± 0.01 at 750 nm, and 0.92 ± 0.01 at 900 nm (Figure 2).

[00242] Crosslinking resulted in decreased transmittance of ultraviolet light (0.55 ± 0.06 at 358 nm, $p = 0.03$ when compared to data before crosslinking (0.78 ± 0.04 at 420 nm), and greater transmittance of visible light (0.92 ± 0.02 at 570 nm, $p = 0.04$, 0.95 ± 0.01 at 750 nm, $p = 0.01$, and 0.95 ± 0.01 at 900 nm, $p = 0.01$).

[00243] OCT imaging confirmed that the scaffold maintained a corneal shape with a central thickness of 502 μm and reflectivity profile comparable to that of a normal human cornea (Figure 3). The mean water content of the scaffold was $88.7 \pm 0.7\%$, w/w (mean $\pm$ SD, average of 3 scaffolds), and was $83.2 \pm 2.8\%$, w/w ($p = 0.07$) after collagen crosslinking.

Example 13: Results: scaffold degradation *in vitro*

[00244] Fifty percent of the original weight of uncrosslinked scaffolds remained after 5 days compared to complete degradation of Oasis 72 hour corneal collagen shields after 3 days (Figure 4).

[00245] Collagen crosslinking significantly improved the stability of the scaffold, with 72% of the original weight of crosslinked scaffolds remaining after 14 days. The difference between the crosslinked and uncrosslinked scaffolds was statistically significant at all time points ($p < 0.01$).

Example 14: Results: light microscopy and scanning electron microscopy of scaffold

[00246] Light microscopy demonstrated that the collagen concentrated by centrifugation was arranged in multiple lamellae, seen at the cut edge; a high degree of alignment was observed in a collagen piece concentrated by centrifugation then dehydrated (Figure 5).

[00247] Scanning electron microscopy of the scaffold showed that the surface of the scaffolds was amorphous and that the scaffold was composed of multiple lamellae on cross-section (Figures 6 and 7).

Example 15: Results: stress, strain, and stiffness measurements for scaffold

[00248] The experimental set up is represented schematically in Figure 11. A representative stack of 2D OCT cross-sections is shown in Figure 12, for an unloaded collagen scaffold sample. Figure 13 shows representative cyclic-load curves from the various samples for 0.1 %. s^{-1} . Figure 14 shows the load response curve for the same samples when strained to failure at 0.5 %. s^{-1} .

[00249] Figure 13 shows representative cyclic load curves from the various samples for 0.1 %. s^{-1} . For the range of strains explored in the cyclic loading tests, a linear fit to the data was sufficient to estimate the stiffness of the sample. Linear fits with an r^2 of less than 0.95 were not included in subsequent analyses. Those fits with r^2 less than 0.95 were typically from corneal stromal samples at extremely low strain cycles. This is probably because stress relaxation behaviour in living tissue during idle periods is greater than in engineered scaffolds. Figure 14 shows strain to failure response curves from the various samples for 0.5 %. s^{-1} . This shows how the strains applied during the strain to failure experiments were sufficient to expose observable non linearity in the stress response, especially for the samples derived from the anterior and posterior stroma.

[00250] It is clear from these experiments, that Young's modulus is only a rough guide for the stiffness response of living tissues. See Figure 14. As previous literature in this domain has been limited to this approximation, so to we limit our analysis for the purpose of comparison to extant data. To obtain estimates of Young's modulus for the strain-to-failure response of the living tissue samples, the regions of the stress-strain response were selected immediately prior to failure as depicted in Figure 14. The first derivative of stress was used to extend the fitting region as far as possible while staying within ± 10 % of the value of slope before failure.

[00251] Figure 15 shows a box plot of the stiffness computed from cyclic loading data. As can be seen from this data, EDC crosslinked collagen scaffold samples showed substantially

higher stiffness than the anterior or posterior stromal samples (greater than two-fold increase; Figure 15). Increased stiffness was also observed for genipin and UV-riboflavin crosslinked collagen scaffold samples (Figure 15). It was noted that even the uncrosslinked scaffolds were generally stiffer than the stromal samples tested, over the range of stress and strain rates tested (Figure 15).

[00252] It is interesting to compare the Figure 15 data with the data shown in Figures 16 and 17, obtained from the strain to failure tests. The tests for Figure 16 were performed at significantly higher strain rates (5 fold) than those used in the cyclic loading tests. In these tests, EDC crosslinked collagen scaffold samples showed higher stiffness than the anterior or posterior stromal samples (Figure 16), although this difference was somewhat less than that observed for lower strain rates (compare Figures 16 and 17). Genipin and UV-riboflavin crosslinked collagen scaffold samples did not show a significant difference from anterior stromal samples (Figure 16). This data reinforces the results from the plot of strain to failure tests. That is, the results for collagen scaffold samples depend on the imposed strain rate. See also, Table 1 below.

Table 1: Summary of tensile strength assessments

	Young's Modulus (MPa) at strain rate 0.1%/s Mean $\pm$ std	Young's Modulus (MPa) at strain rate 0.5%/s Mean $\pm$ std	Ultimate tensile strength (MPa) at strain rate 0.5%/s Mean $\pm$ std
Anterior stroma	1.16 $\pm$ 1.42	9.72 $\pm$ 7.53	1.53 $\pm$ 0.86
Posterior stroma	1.41 $\pm$ 1.35	2.04 $\pm$ 1.53	0.55 $\pm$ 0.22
Uncrosslinked	4.92 $\pm$ 0.73	4.83 $\pm$ 1.39	1.23 $\pm$ 0.27
UVA + Riboflavin	9.68 $\pm$ 1.63	13.47 $\pm$ 5.73	2.21 $\pm$ 0.70
EDC	26.3 $\pm$ 11.83	34.89 $\pm$ 22.14	5.38 $\pm$ 2.48
Genipin	12.70 $\pm$ 2.11	12.32 $\pm$ 5.35	1.96 $\pm$ 0.034

[00253] The stiffness values plotted in Figure 16 and Figure 17 were then compared across sample types, strains and strain rates via pairwise ANOVA. In order to examine the differences further, a t-test was conducted on all pairwise comparisons. The results of this comparison are shown in Table 2 for cyclic loading tests and Table 3 for strain-to-failure tests. A comparison of the ultimate tensile strengths of the various samples is shown in Table 4. For Table 2, Table 3, and Table 4, * = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$. See also Figure 18. Because the data

did not appear to be normally distributed, the log transform was applied prior to the analysis. Consequently, the results represent ratios of means rather than differences. Of particular interest are the effects of crosslinking on the engineered scaffold apparent in both types of tests. Increases in stiffness with crosslinking were statistically significant. From the testing, it was concluded that EDC crosslinking provided improved stiffness compared to UVA + riboflavin or genipin crosslinked compositions.

[00254] To determine the relative effects of crosslinking, pairwise t-tests were performed between all cross-linking groups while controlling for the effects of the strain and strain-rate, the subsequent results are presented as ratios of mean tensile strength in Table 4A. For Table 4A, $p < 0.01 = **$ and $p < 0.0001 = ****$. Ratios are presented as columns over rows, e.g., the log-transformed mean tensile strength of UV/riboflavin crosslinked gel/uncrosslinked gel equals 1.824, and the differences between UV/riboflavin crosslinked and uncrosslinked gels were statistically significant at $**p < 0.01$. The ultimate tensile strength of uncrosslinked scaffolds (average 1.23 MPa) falls between the values of the anterior (1.53 MPa) and posterior (0.55 MPa) stroma. Crosslinking using UV/riboflavin (2.21 MPa), genipin (1.96 MPa), and EDC (5.38 MPa) increased the ultimate tensile strength of scaffolds. All were statistically significant compared to uncrosslinked scaffolds. UV/riboflavin crosslinking produced an ultimate tensile strength that was significantly different from EDC, but not genipin.

Table 2: Ratios of maximum stiffness from cyclic loading experiments

	UnXL	UV+R	Gen	EDC	Ant	Post	Strain	Strain rate
Uncrosslinked	1.000	1.824	**	****	4.796	****	0.105	****
UV+Riboflavin		1.000			2.630	****	0.057	****
Genipin			1.000		1.866	****	0.041	****
EDC					1.000	****	0.022	****
Anterior						1.596	0.773	*
Posterior						1.000	0.773	*

Table 3: Ratios of maximum stiffness from strain-to-failure experiments

	UnXL	UV+R	Gen	EDC	Ant	Post	Strain rate
Uncrosslinked	1.000	2.738	2.498	6.643	*	1.724	0.333
UV+Riboflavin		1.000	0.913	2.426		0.630	0.122
Genipin			1.000	2.659		0.690	0.133
EDC				1.000		0.260	0.050
Anterior					1.000	0.193	*
Posterior						1.000	*

Table 4: Ratios of ultimate tensile stress from strain-to-failure experiments

	UnXL	UV+R	Gen	EDC	Ant	Post	Strain rate
Uncrosslinked	1.000	2.738	2.498	6.643	*	1.724	0.333
UV+Riboflavin		1.000	0.913	2.426		0.630	0.122
Genipin			1.000	2.659		0.690	0.133
EDC				1.000		0.260	0.050
Anterior					1.000	0.193	*
Posterior						1.000	*

Table 4A: Ratios of means of tensile strength from pairwise comparison

	Un-cross-linked	UVA + Riboflavin	Genipin	EDC	Anterior stroma	Posterior stroma
Un-cross-linked	1.000	1.824	** 2.570	****4.796	0.105	**** 0.167
UVA + Riboflavin		1.000	1.409	2.630	0.057	**** 0.092
Genipin			1.000	1.866	0.041	**** 0.065
EDC				1.000	0.022	**** 0.035
Anterior stroma					1.000	1.596
Posterior stroma						1.000

[00255] Estimates of stress which form the grounds for estimates of stiffness are highly dependent on the measured cross sectional area. As such, mis-classification of image pixels has the potential to introduce errors into the measurement. A comparison was made between areas detected by the classifier, with area selected by a human operator for the same image. This test was performed across $N = 20$ images from 5 samples including two tissue samples. The classifier consistently over estimated area by between 15% and 25%, when compared with a human operator, a relatively large effect. The systematic nature of the effect, coupled with the significance of the p -values, especially in the cyclic loading data, should leave the relationships between samples largely unchanged.

[00256] Due to the size, and constitutive properties of the samples under test, it was difficult to ensure a consistent cross section when dissecting. The difference in area could be as high as 20% between largest and smallest cross sections (see Figure 19). The relationships between ultimate tensile stresses in Table 4 are computed based on an assumption that a minimum cross sectional area is an appropriate reference for failure stress computations. A more comprehensive treatment would be to mesh the OCT image stack and use the mesh to construct a finite element model. The recorded force could then be applied as a boundary condition to the model, and the predicated response compared with the measured geometry change to aid in formulating a constitutive model of the scaffolds.

Example 16: Results: epithelial and keratocyte cell growth on the scaffold

[00257] The scaffold supported epithelial cell growth on the surface and keratocyte growth both on the surface and within the scaffold itself (Figures 8A-10C). Light microscopy showed that keratocytes from porcine keratocyte holoclones covered the surface and infiltrated the interior of the scaffold from 17 days in culture (Figures 8A-8B).

[00258] Confocal microscopy of cross-sections of the scaffold after 42 days of culture showed that the cells covering the surface of the scaffold were fibroblastic and immuno-positive for α -SMA, while the cells within the scaffold had retained the keratocyte phenotype, being immuno-positive for keratocan (Figure 8C and Figure 9).

[00259] Epithelial cells from a human donor limbal rim migrated and fully covered the surface of a scaffold in multiple layers after 28 days of culture. Immunohistochemistry showed that the cells were immuno-positive for cytokeratin, a marker for epithelial cells (Figure 8D).

[00260] Keratocytes were visible throughout the depth of the scaffold (Figure 10A-10B), and positioned in a lamellar arrangement (Figure 10C). Keratocytes were observed to spread horizontally up to approximately 600 microns from the injection site over the course of 21 days (Figures 10A-10B).

Example 17: Experimental observations for synthesis and testing of collagen scaffold

[00261] Polarised light and electron transmission microscopy studies have shown that in many biological tissues, the three-dimensional arrangement of collagen fibrillar networks follow the same spatial distribution as those described in certain liquid crystals (Martin et al., 1999). This has been observed for compact bone, tendon, skin, and cornea. It has also been established that soluble collagen exhibits liquid crystalline properties *in vitro*. Dilute collagen solutions are isotropic, i.e., the molecules take up random orientations in the fluid. However, beyond a critical concentration (thought to be 80-85 mg/ml) lyotropic liquid-crystal phases appear (Belamie et al., 2006). This spontaneous development of order leads to the formation of strong birefringence (Murthy, 1984). The production of liquid crystalline collagen is therefore desirable for the development of scaffolds that mimic biological tissues.

[00262] The inventors were able to create a cornea shaped piece of scaffold from type I collagen with a thickness (502 μm) comparable to that of the human cornea (Paul et al., 2008). Bovine corneal stroma has a water content of 75-85%, w/w (Doughty, 2001; Castoro et al., 1988) and human corneas have a water content of approximately 80%, w/w (Pircher et al., 2002). The water content of the scaffold (88.7%, w/w before crosslinking, and 83.2%, w/w after crosslinking) was slightly higher than that of the cornea, possibly related to the lack of proteoglycans and endothelial cell pump regulation of hydration.

[00263] The transmittance of the uncrosslinked scaffold in the UV range (0.72 at 358 nm) is comparable to that of the human cornea, as reported in previous studies (0.70 at 358 nm at age younger than 45 (Beems and Best, 1980), 0.54 at age older than 45 (Beems and Best, 1980), and 0.72 for all ages (van den Berg and Tan, 1994)). The transmittance in the visible light range, e.g., 0.88 at 570 nm and 0.92 at 900 nm, is slightly less than that of the human cornea (0.91-0.92 at 570 nm and 0.96 at 900 nm at all ages (Beems and Best, 1980; van den Berg and Tan, 1994)). Crosslinking of the scaffold decreased transmittance of UV light from 0.72 to 0.55 at 358 nm, akin to the effect of aging (Beems and Best, 1980), and increased transmittance in the visible light range from 0.88 to 0.92 at 570 nm, and from 0.92 to 0.95 at 900 nm, comparable

with the human cornea. This is also consistent with the observation of increased transparency of porcine corneas after UV/riboflavin crosslinking (Wollensak, 2007).

[00264] During the degradation assay, a small increase in wet weight (% weight remaining) was initially seen in the crosslinked scaffolds and the corneal shield (Figure 4). We attribute this to continued osmotic swelling, despite pre-soaking the samples in a control solution for 3 days before the degradation assay. Degradation of the uncrosslinked scaffold was slow compared to the 72 hour corneal shield. The corneal shield was fully degraded after 3 days, whereas more than 60% of the uncrosslinked scaffold remained and 100% of the crosslinked scaffold remained at this time point. Collagen crosslinking has previously been shown to increase resistance to collagenase degradation (Meek and Hayes, 2013). Therefore the scaffold was relatively stable and can be further stabilised by collagen crosslinking.

[00265] Light microscopy, scanning and transmission electron microscopy showed that the scaffold was composed of layers of collagen, consistent with the lamellar organisation of collagen in the corneal stroma (Komai and Ushiki, 1991). Therefore this substrate has the potential to be re-populated by keratocytes in a layered organisation as in native cornea. Collagen crosslinking significantly improved the mechanical strength of the scaffold, with Young's Modulus and ultimate tensile strength exceeding that of the anterior and posterior human corneal stroma. This was not surprising as UV/riboflavin crosslinking of human corneas has been shown to increase the biomechanical strength of the cornea by 300% (Wollensak et al., 2003). Moreover, the scaffold supported re-epithelialisation and keratocyte cellularisation, demonstrating superior biological activity compared to artificial materials used in artificial corneas, which typically do not support cellularisation.

[00266] In summary, the disclosed collagen scaffolds show several advantages over available compositions and matrices. The scaffold production methods are rapid and simple. The production technique enables thicker scaffold structures. The scaffolds have a high tensile strength that is comparable to native tissue. The scaffolds also have an aligned and lamellar collagen structure, and retain a high degree of transparency. The scaffolds are mouldable and adaptable; scaffold size, shape, and thickness can be customised for a particular application or a specific subject to be treated. Further, they are expected to maintain the desired shape *in vivo*. Finally, the scaffolds are biocompatible and support cell growth onto and into the three dimensional structure.

Example 18: Overview of experiments testing keratocyte seeding of collagen scaffold

[00267] The collagen scaffold described herein is a useful product for human tissue repair. The experiments outlined above have confirmed that the scaffold has a lamellar structure, is biocompatible, and has viscoelastic properties and a high ultimate tensile strength that is comparable to that of human corneal tissue (see also, Zhang et al., 2015). Importantly, the strength of the scaffold enables it to be easily sutured.

[00268] Biological scaffolds may act as templates for tissue regeneration, supporting corneal remodelling by a recipient's endogenous corneal cells. Alternatively, scaffolds can be seeded with appropriate cells, e.g., keratocytes, keratocyte progenitor cells, and stromal stem cells, *in vitro* before transplantation (Patel et al., 2013; Wu et al., 2014). Few studies have examined the migration dynamics or phenotypic maintenance of infiltrating quiescent keratocytes into biological scaffolds over long periods of time. This is a key factor, since keratocytes produce collagen and proteoglycans, which are required for optimal corneal remodelling (Funderburgh et al., 2003; Jester et al., 2008). Furthermore, maintenance of the quiescent keratocyte phenotype is necessary to prevent excessive scaffold contraction, caused by differentiation into the contractile fibroblastic or myofibroblastic phenotypes (Meshel et al., 2005; Jester et al., 1995).

[00269] The aim of these experiments was to investigate the migration and phenotype of keratocytes implanted into the collagen scaffold and to determine if a biomimetic distribution of quiescent keratocytes can be established, either as a potential pre-implantation step or as an indirect indicator of potential *in vivo* infiltration by endogenous keratocytes.

Example 19: Scaffold formation, cell injection, and culturing in hydrated scaffolds

[00270] Formation of collagen scaffolds was carried out as in Example 2, with the following modifications. Prior to the initial hydration step, each piece of collagen gel was placed on a glass slide. The flattened pieces were removed from the glass slides. The hydration step was then carried out via centrifugation as noted in Example 2. In the current study an 8 mm x 12 mm (height x internal diameter) plastic ring mould was used to form the hydrated scaffolds. After the neutralisation step as noted in Example 2, the scaffolds were removed from the mould and placed into Milli-Q water and stored at 4°C prior to cell injection.

[00271] Isolation of primary porcine keratocytes and culturing to form keratocyte spheroids was carried out as described in Example 10.

[00272] Scaffolds were cut in half with a scalpel. One half was placed in culture medium and used as a cell free control. The other half was placed on a petri dish and a disposable 1.5 mm wide microvitrectomy blade was used to puncture two vertical channels through approximately 90% of the scaffold thickness. In one scaffold only a single channel was created. Keratocyte spheroids were extracted from culture trays with a 0-200 μ l pipette set to a volume of 100 μ l. Spheroids were pipetted ('injected') into the channel. Pipetting was repeated until each channel was maximally filled with spheroids. Cell injected and cell free control scaffolds were cultured in standard 12-well polystyrene culture trays, with each well containing 2 ml of Advanced DMEM (Gibco®, Grand Island, NY, USA), supplemented with 1% GlutaMAX (Gibco®, Grand Island, NY, USA), 1% antibiotic/antimycotic (Gibco®, Grand Island, NY, USA), and 25 ng/ml recombinant human IGF-II (Gibco®, Grand Island, NY, USA). The culture medium was replaced twice weekly and culture trays were kept in a humidified incubator at 37°C, with 5% CO₂.

Example 20: Microscopy, rate of cell migration, and scaffold contraction measurements

[00273] Scaffolds and keratocytes were monitored using a Leica DMIL IL LED inverted microscope (Leica Microsystems, Wetzlar, Germany). Digital images for examining keratocyte migration and scaffold thickness were acquired using a Leica MC120 HD microscope camera (Leica Microsystems, Wetzlar, Germany), controlled by a PC computer running Leica Application Suite version 4.4.0 (Leica Microsystems, Wetzlar, Germany). For scaffold radius measurements, a Zeiss Discovery V20 stereo microscope (Zeiss, Oberkochen, Germany) with darkfield illumination was used. Images were acquired with an attached Nikon Digital Sight DS-5Mc camera (Nikon Corporation, Tokyo, Japan) controlled by a PC computer running NIS Elements BR software (version 4.3, Nikon Instruments Incorporated, Tokyo, Japan).

[00274] Rate of cell migration was calculated in micrometres per day (μ m/day) by measuring the distance from the edge of the injection site to the cell that had migrated furthest (taking into account cells at all depths through the scaffold), then dividing by the number of days post-injection. Measurements were made from acquired images with Leica Application Suite (version 4.4.0, Leica Microsystems, Wetzlar, Germany) or Photoshop CC 2014 (Adobe Systems Incorporated, San Jose, CA, USA).

[00275] Images of scaffolds were acquired every 2-5 days for thickness and radius measurements. To measure scaffold thickness ~ 95% of the media was removed from the cell culture wells, leaving just enough media to keep the base of the scaffolds wet. Scaffolds were

oriented cut surface down against the side of the well, then the culture tray was transferred to a Leica DMIL IL LED inverted microscope (Leica Microsystems, Wetzlar, Germany). 'Landmarks' in the form of bubbles, ripples, or folds were identified along the length of the cut surface of the scaffold. Due to the size of the scaffolds being larger than the maximum field of view with the lowest magnification objective lens (4X lens, 40X total magnification), multiple images were taken along the length of the scaffolds to capture all of the landmarks. Images were montaged together and the width of the cut surface (i.e., scaffold thickness) was measured using Adobe Photoshop CC 2014 (Adobe Systems Incorporated, San Jose, CA, USA).

[00276] Scaffold radii were measured on acquired images using the N points circle radius tool in NIS Elements BR software (version 4.30, Nikon Instruments Incorporated, Tokyo, Japan). Scaffold measurements were imported into Microsoft Excel 2013 software (Microsoft, Redmond, WA, USA) for statistical analysis and graphing. Mean thickness and mean radius were calculated for each time point, normalised to the day 0 mean (prior to keratocyte injection), and expressed as a percentage. Mean thicknesses and mean radii were compared between scaffolds with and without cells at each individual time point using unpaired t-tests. A *p*-value less than 0.05 was used to denote statistical significance. Normalised means were graphed with the standard error of the mean used as error bars.

Example 21: Confocal imaging of live keratocytes in scaffolds

[00277] Confocal microscopy was used to image keratocytes in the three dimensional volume of the hydrated scaffolds. Scaffolds were placed in culture medium containing 2 μM Calcein-AM and incubated for 1 h at 37°C and 5% CO₂. Scaffolds were then transferred into a 35 mm FluoroDish™ with 23 mm well (World Precision Instruments, Sarasota, FL, USA) and covered with just enough (calcein free) media to keep them wet, but not enough to completely cover them. This was done so that scaffold movement could be avoided when the microscope stage was moved. The FluoroDish™ was placed on a Zeiss LSM 710 inverted confocal microscope equipped with a Zeiss PM S1 stage-top incubator (Zeiss, Oberkochen, Germany), set to 37°C and 5% CO₂. Confocal image stacks were obtained using ZEN 2010 software (Zeiss, Oberkochen, Germany). Maximum projections and orthogonal projections were created using ImageJ software (version 1.5g, National Institutes of Health USA; Schneider et al., 2012). Scaffolds were transferred back into standard incubation medium after imaging and returned to the cell incubator for continued culturing.

Example 22: Fixation, labelling, and imaging of keratocytes in scaffolds and porcine cornea

[00278] Whole porcine eyes were sterilised by immersion in 4% povidone-iodine for 2 min, followed by 1 min in 4% sodium thiosulphate. Eyes were washed in PBS then corneas dissected out. Corneal epithelial cells were removed by scraping using a microkeratome and a ~8 mm x ~8 mm piece of central cornea was cut with a scalpel for fixation and antibody labelling.

[00279] Scaffolds and native porcine cornea pieces were transferred into a 5 ml plastic specimen vial. Next, 4 ml of 4% paraformaldehyde (PFA) in PBS (preheated to 37°C) was added and the scaffolds incubated 1.5 h at 37°C. PFA was removed and scaffolds were washed 3 x 30 min in 4 ml of PBS. Scaffolds were stored overnight in 4 ml of fresh PBS at 4°C. PBS was replaced with 4 ml of 0.5% Triton™ X-100 in PBS and incubated for 30 min to permeabilise cells. The 0.5% Triton™ X-100 in PBS was removed and scaffolds were rinsed 3 x 1 min with 4 ml of PBS. Scaffolds were incubated for 2 x 20 min and 1 x 40 min in 4 ml of PBS. PBS was removed and scaffolds incubated in 4 ml of blocking solution (2% Triton™ X-100, 10% normal goat serum in PBS) for 3 h. Blocking solution was removed and scaffolds were incubated in 2 ml of primary antibody solution, comprising 0.5% Triton™ X-100, 10% normal goat serum, and primary antibodies, in PBS overnight at 4°C. Primary antibodies used were rabbit anti-keratocan (Santa Cruz, Dallas, Texas, sc-66941, 1:25), as a marker for keratocytes, and mouse anti-alpha smooth muscle actin primary antibody (α -SMA, Leica Biosystems, Newcastle, UK, NCL-SMA, 1:40), as a marker for myofibroblasts.

[00280] Primary antibody solution was removed and scaffolds washed 3 x 20 min in 4 ml of PBS. Scaffolds were incubated for 3 h in 3 ml of secondary antibody solution, comprising goat anti-rabbit Alexa488 secondary antibody (Life Technologies, Pleasanton, CA, USA, A11011, 1:100) and goat anti-mouse Alexa568 secondary antibody (Life Technologies, Pleasanton, CA, USA, A10680, 1:100). Scaffolds were rinsed twice then washed 2 x 20 min in 4 ml PBS. Keratocyte nuclei were labelled by incubating scaffolds for 30 min in PBS containing DAPI (Life Technologies, Carlsbad, CA, USA, 1:10,000). Scaffolds were washed for 20 min in PBS, immediately followed by confocal imaging (see below). If required, fixed antibody-labelled scaffolds were stored in PBS at 4°C.

[00281] Fixed antibody-labelled scaffolds or cornea pieces were transferred into 35 mm FluoroDish™ with 23 mm well (World Precision Instruments, Sarasota, FL, USA) and covered with just enough PBS to keep them wet, but not enough to completely cover them. This prevented sample movement when the microscope stage was moved. The FluoroDish™ was

placed on a Zeiss LSM 710 (Zeiss, Oberkochen, Germany) inverted confocal microscope and image stacks were obtained using ZEN 2012 software (Zeiss, Oberkochen, Germany).

Example 23: Results: cell injection of scaffolds and keratocyte migration

[00282] Figure 20A shows an example of a hydrated collagen scaffold injected with keratocyte spheroids. Figure 20B shows an example of injected keratocyte spheroids in a scaffold viewed in cross-section.

[00283] Within 24 hours of injection, small dendritic cell processes were observed projecting from the edge of the injection sites into the surrounding scaffold (Figure 21A). Keratocytes initially migrated in close contact with other keratocytes, due to the high density of cells in and around the injection site. However individual keratocytes were discernible migrating away from the injection site within 3-5 days (Figure 21B). Keratocyte migration distance varied around the injection site, with depth through the scaffold, and between scaffolds, therefore migration was compared by calculating the maximum rate of migration per injection site in micrometres per day ($\mu\text{m}/\text{day}$). Figure 21C shows an example with a maximum migration rate of $57 \mu\text{m}/\text{day}$. Calculating the maximum migration rate for 10 different injection sites (across 7 different scaffolds) yielded a range of $29\text{-}72 \mu\text{m}/\text{day}$, with a mean of $50 \pm 12 \mu\text{m}/\text{day}$.

[00284] To allow better visualisation of their three dimensional distribution in the scaffolds, keratocytes were loaded with calcein and imaged using a confocal microscope. With a 10X dry objective lens, the calcein fluorescence signal in keratocytes was strong and allowed clear visualisation of keratocyte distribution and morphology (Figure 22). Figure 22A shows a maximum projection through a confocal image stack. In this experiment, keratocytes migrated radially away from the injection site over 21 days. A maximum projection of orthogonal (Z-X axis) sections, generated from the same confocal image stack, strongly suggests that the keratocytes were migrating in discrete layers or sheets (Figure 22B). The pattern and distance of keratocyte migration varied in both the X-Y axis and the Z-axis, as shown by images acquired at different depths through the scaffold (Figures 22C, D, and E).

Example 24: Results: keratocyte morphology and phenotype, and scaffold contraction

[00285] Keratocyte morphology was clearly visible using bright field microscopy (Figure 23A) and even clearer when keratocytes were loaded with calcein and viewed with a confocal microscope (Figure 23B). As keratocytes moved away from the injection site 'columns' of cells

could often be discerned, typically with one or two keratocytes at the front of the column establishing a pathway through the scaffold (Figure 23B). These leading keratocytes (Figure 23B) displayed a distinctive polar morphology with multiple dendritic processes extending predominantly in the direction of the individual keratocyte's migration.

[00286] After at least 9 weeks post-injection, keratocytes took on a quiescent phenotype, characterised by development of a stellate morphology, with a more rounded cell body, and increased intercellular dendritic processes (Figure 24C). This morphology was similar to that of keratocytes in intact porcine corneas (Figure 24A). Labelling with an antibody against α -SMA, provided further confirmation of the quiescent phenotype (Figure 24D), although there was a low level of diffuse α -SMA in all keratocytes, regardless of whether they were within scaffolds or intact corneas (Figures 24B, D, and E). Keratocytes on the surface of scaffolds were predominantly confluent, with a low level of diffuse α -SMA labelling (Figure 24F). A minority of these surface keratocytes displayed strong α -SMA labelling in the form of stress fibres (Figure 24F, white arrows), suggesting myofibroblastic differentiation. Stress fibres were not visible in any keratocytes fully embedded within hydrated collagen scaffolds or intact porcine corneas.

[00287] Scaffolds with cells displayed a decrease in thickness of 36% and a decrease in radius of 5% at 5 days post-injection. For scaffolds without cells mean thickness decreased by 32% and mean radius by 7% at 5 days post-injection. Scaffold contraction then slowed and levelled off, so that by 46 days post injection scaffolds with cells displayed a reduction in mean scaffold thickness of 45% and mean scaffold radius of 12%, while scaffolds without cells displayed a reduction in mean thickness of 40% and mean radius of 10% (Figure 25). There was no significant difference in mean thickness or radius between scaffolds injected with keratocytes and control acellular scaffolds at each of 12 time points measured across 46 days ($p>0.05$ for each time point).

[00288] The results provide further support for the utility of the collagen scaffold in tissue repair and augmentation. In these experiments, cells were introduced into the scaffold after the neutralisation step (i.e., before dehydration). This facilitated injection of the cells into the scaffolding. In addition, it was also possible to implant an acellular scaffold and obtain sufficient cell populations within the scaffold. With such implantation, the cells were able to infiltrate the scaffold while also maintaining a quiescent keratocyte phenotype.

[00289] This study confirms that primary keratocytes can migrate between type I collagen lamellae in a preformed hydrated scaffold to establish a distribution similar to native porcine

and human corneas. The noted observations suggest that the rate of cell migration could be increased when scaling the production of laboratory grown scaffold for clinical use. For example, pro-migratory regulatory factors could be added to the scaffold *in vitro* or *in vivo* to produce a diffuse and even distribution of keratocytes in the scaffold. Alternatively, the cell free scaffold could be surgically implanted and the scaffold recipient's endogenous keratocytes could repopulate the cell free scaffold *in vivo* over time, without fibroblastic or myofibroblastic differentiation.

[00290] Notably, cells cultured in engineered 3D microenvironments have been shown to better represent *in vivo* cellular behaviour (in terms of viability, proliferation, gene expression, and differentiation) than cells cultured in 2D configurations. These differences have been attributed to differences in the distribution of cell-cell and cell-extracellular matrix interactions, which alter cell morphology, signalling mechanisms and subsequent cell function. The differences in cell phenotype observed within the scaffold compared to on its surface may in part be explained by the 3D and 2D microenvironments respectively. The organisation of the 3D micro-environment is also known to play an important role in extracellular matrix deposition and remodelling. In the case of corneal stroma, collagen fibril diameter and alignment are known to affect myofibroblast differentiation, keratocyte regeneration, and matrix synthesis. It is therefore anticipated that the aligned and lamellar structure of the scaffold will encourage appropriate tissue remodelling by resident keratocytes.

Example 25: *In vivo* testing of collagen scaffold for anterior lamellar keratoplasty

[00291] The collagen scaffold was synthesised as described in Example 2, with the following modifications. Medical grade bovine collagen was used in lieu of rat collagen. Centrifugal ultrafiltration was performed by placing 10 mL of 5 mg/mL type I bovine collagen (Symatase, Chaponost, France) in a Vivaspın® Turbo 15 unit (Sartorius AG, Goettingen, Germany) with a 10 kDa molecular weight cut off polyethersulfone (PES) membrane. In addition, prior to the initial hydration step, each piece of collagen gel was placed on a glass slide. Another glass slide was placed on top of each piece and pressed down to flatten to a thickness of approximately 0.5-1 mm. The flattened pieces were removed from the glass slides. The hydration step was then carried out via centrifugation as noted in Example 2, and the remaining synthesis steps of Example 2 were followed.

[00292] Anterior lamellar keratoplasty was performed on a rabbit cornea as follows. The rabbit was anaesthetised. The operative eye and surrounding eyelids were cleaned with 5%

povidine iodine, and oxybuprocaine 0.4% eye drops were instilled. A sterile drape was applied to the operative field and after cutting a horizontal slit in the drape, a speculum was inserted to separate the eyelids. A 6-0 chromic gut suture was placed underneath the medial rectus muscle to stabilise the eye. A shortcut angled blade was used to make a partial depth incision in the superio-temporal limbus. A lamellar dissector was then inserted at the base of the incision and advanced towards the central cornea to create a plane of separation. A 6.5 mm Barron vacuum trephine was used to make a circular incision in the anterior cornea. A 15 degree blade and corneal scissors were used to dissect and remove the anterior corneal button. A 6.5 mm donor trephine was used to cut the scaffold to form a donor button which was subsequently sutured in place using eight 10/0 nylon sutures. The eye was treated with chloramphenicol and Pred Forte® eye drops twice daily for 16 days. The corneal sutures were removed 14 days post-operatively.

[00293] Figure 26A shows the treated eye on the day of surgery. Figures 26B and C show the treated eye 16 days following surgery. At 16 days following surgery, the transplanted scaffold remained transparent and was fully epithelialised. There were no signs of infection of the surgical site, and no signs of rejection of the scaffold material. The collagen scaffold showed no signs of deterioration.

Example 26: Overview of synthesis and testing of collagen scaffold for tendon repair

[00294] Tendon injuries are an increasingly common clinical problem occurring in otherwise healthy, active people and are primarily caused by overuse or trauma. Tears of the rotator cuff tendon in the shoulder are a common clinical problem, with full thickness tears present in approximately 22% of the general population, an incidence that increases with age, with more than 54% of those over 60 years of age having partial or complete tears of the rotator cuff. Due to the high incidence of rotator cuff tears and the increasingly aged population, there has been a near four-fold increase in the number of surgical interventions required over the past 10 years.

[00295] Tendon injuries heal poorly with the formation of functionally compromised fibrotic scar tissue. Even after surgical repair, healing is suboptimal. In human patients over the age of 65, only 43% of patients showed evidence of some healing 18 months after arthroscopic repair of full thickness rotator cuff tears. Furthermore, re-tear rates are reported to be as high as 69% for repairs of large full thickness rotator cuff tears. These injuries affect

patient quality of life and become significant financial issues - recurrent rotator cuff tears costing are estimated to cost approximately NZ\$100 million per annum.

[00296] Biological augmentation with tissue engineered grafts has been suggested as a method for improving healing outcomes of such injuries and could significantly reduce the unacceptably high re-tear rates associated with rotator cuff tears. There are a small number of materials commercially available for this, however, available data on their efficacy is poor, and there is a clear need for new and better alternatives.

[00297] The collagen scaffold noted herein has evident utility for tissue augmentation. Therefore, *in vitro* testing was carried out to assess immunogenicity for tendon tissue, so as to determine the likelihood that this scaffold will cause an immune response *in vivo*. It was then assessed the scaffold in an *in vivo* model of rotator cuff repair, designed to mimic the current clinical practice carried out in orthopaedic surgeries globally.

Example 27: Scaffold preparation for tendon repair

[00298] The collagen scaffold was prepared as follows. Centrifugal ultrafiltration was performed by placing 10 mL of 5 mg/mL Cultrex® 3D culture matrix rat collagen I (Trevigen, Gaithersburg, Maryland, USA) in a Vivaspın® Turbo 15 unit (Sartorius AG, Goettingen, Germany) with a 10 kDa molecular weight cut off (MWCO) polyethersulfone (PES) membrane. The solution was spun in a centrifuge unit (Sigma 3-16KL) at 4°C at 4500 rpm for 22 hours (final volume 0.3 mL; concentration 125 mg/mL).

[00299] The resulting firm concentrated collagen gel was divided into 6 pieces. Each piece was placed on a glass slide and neutralised with 4 µl of NaOH for each piece. A glass cover slip was placed on top of each piece and pressed down to flatten to the desired thickness of approximately 2 mm. The scaffolds were then placed in a humid chamber at 37°C for at least 4 hours. They were then placed in a dry incubator at 37°C for at least 12 h to allow complete dehydration. Prior to using the scaffolds, they were rehydrated by immersion in Milli-Q water for 4 hours.

Example 28: *In vitro* and *in vivo* testing of scaffold for tendon repair

[00300] *In vitro* testing was performed as follows. The immunogenicity of the collagen scaffold was assessed in an assay for pre-macrophage cell activation. Human THP-1 cells (1.5 million per well) were seeded in 24-well plates containing either Vicryl® deep tissue suture material (clinical control) or a 1.9 cm diameter disk of the collagen scaffold in RPMI medium

with 10% FBS. Cells were harvested following 24 and 48 hours incubation. The expression of pro- and anti-inflammatory marker genes associated with macrophage activation was assessed by real-time PCR analysis.

[00301] *In vivo* testing was performed as follows. Surgical procedures were carried out in accordance with the requirements set by the Institutional Animal Ethics Committee. Sexually mature, male Sprague-Dawley rats (n=23), weighing over 300 g, underwent surgery. Rats were checked for general health, weight-matched and distributed to three groups: 1) sham surgery (approach to supraspinatus only; n=3); 2) unaugmented control (single row supraspinatus repair; n=10); 3) intervention group (single row repair augmented with collagen scaffold; n=10).

[00302] At least 2 hours prior to surgery, rats received a subcutaneous injection of the non-steroidal anti-inflammatory drug (NSAID) rimadyl (10 µL/g). For anaesthetic induction, rats were placed in a sealed rodent induction box with 5% isoflurane and 2L O₂. Anaesthesia was maintained with 2.5% isoflurane and 2L O₂ through a specialised nose cone. A 2.5 mL subcutaneous injection of normal saline was administered immediately after induction.

[00303] The forelimb was prepared by shaving and washing with 2% chlorhexidine and 70% ethanol, followed by a sterile drape. An approximately 2 cm longitudinal incision was made, centred over the glenohumeral joint, extending proximally along the belly of supraspinatus and distally along shaft of humerus. The middle belly of deltoid was incised proximally, in line with muscle fibres, down to the lateral aspect of the proximal humeral shaft. The acromio-clavicular joint was incised. The supraspinatus was easily identified through this approach. A 5-0 prolene stay suture was passed through the distal supraspinatus. The tendon was incised at its insertion onto the greater tuberosity of the humerus. The insertion site was debrided of any residual soft tissue.

[00304] In the unaugmented control group, supraspinatus repair was carried out using the 5-0 prolene stay suture, and a modified Mason-Allen suture technique. The cut end of the tendon was approximated to the bony insertion and the suture ends were tied with a surgeon's knot. In the intervention group, the collagen scaffolds (5 x 10 mm) were rehydrated with sterile saline, then overlaid longitudinally on the superficial aspect of the tendon-bone insertion and incorporated into the suture repair.

[00305] Following repair, the deltoid and coraco-acromial arch were lightly approximated with 2 interrupted 2-0 vicryl sutures. Skin closure was carried out with a running subcuticular 4-0 monocryl suture with buried knots at either end. 0.4 mL of Marcain® (1.25 mg/mL solution) local anaesthetic was infiltrated around the operation site after closure.

[00306] Rats were closely monitored immediately following surgery. Once sufficiently recovered from the anaesthetic, the rats were housed singularly and transferred to a warming cabinet for one night. Rimadyl (10 μ L/g) and 2 mL normal saline were administered subcutaneously twice daily for 48 hours post-operatively. Rats were weighed daily and checked for signs of illness, pain, or distress twice daily for the first 48 hours post-operation. Following this, they were weighed and checked once daily until 14 days post-operation. They were then weighed and checked on a weekly basis.

[00307] At 12 weeks post-operatively, rats were humanely sacrificed by CO₂ inhalation. The left supraspinatus tendon, attached to the whole humerus, was immediately excised and either placed in formalin for histological analysis or wrapped in PBS soaked gauze and stored at -20°C for later biomechanical analysis.

Example 29: Biomechanical and histological analysis of tendon tissue

[00308] For biomechanical analysis, excised shoulders were defrosted in a 37°C water bath and kept hydrated with H₂O spray throughout testing. The muscle fibres of the supraspinatus were removed by gentle scraping, leaving only the distal tendon attached to the humerus. Suture material was removed to allow testing of the repaired tendon alone.

[00309] Using a specially modified clamp, the humerus and the tendon were positioned in an Instron machine, with the tendon at 45° relative to the humerus, to apply load in a functional position. Specimens underwent a 10 cycle preconditioning phase (0.1 to 0.5 N at a rate of 1%/s), followed by a stress relaxation phase (6% strain, at a rate of 5%/s (0.575 mm/s), followed by 10 min relaxation), then ramp to failure at a rate of 0.3%/s. Stiffness, Young's modulus of elasticity and ultimate load to failure were then calculated. For each treatment group, five samples were tested.

[00310] For histological analysis, Excised shoulders were fixed in 10% neutral buffered formalin, processed, decalcified in 10% formic acid/5% formaldehyde, embedded in paraffin and 5 μ m thick sections were taken. Sections were stained with haematoxylin and eosin (H&E) and analysed using both transmitted and polarised light microscopy to evaluate healing. A semi-quantitative grading system was used based on collagen fibre density, collagen fibre orientation, quality of healing at bone-tendon interface, vascularity, and presence of inflammatory cells. A total was obtained by combining the scores for these five parameters, with a higher score indicating greater healing (Table 5). A minimum of three slides per repair

were assessed where the tendon-bone interface could be identified. For each treatment group, five samples were tested.

Table 5: Histological scoring chart

	Collagen Fibre Density	Collagen Fibre Orientation	Bone-Tendon Interface	Vascularity	Inflammation
0	None	None	0-24% interdigitation	Abundant Vascular Network	Abundant Inflammatory Cells
1	Low	Disorganised Fibres	25-49% interdigitation	Moderate Vascular Network	Moderate Inflammatory Cells
2	Medium	Moderate Alignment	50-75% interdigitation	Minimal Vascular Network	Minimal Inflammatory Cells
3	High	Highly Aligned	>75% interdigitation	No Vascular Network	No Inflammatory Cells

Example 30: Results: immunogenicity of scaffold

[00311] *In vivo* implantation of tissue engineered materials often results in the activation of a host immune response, resulting in chronic inflammation. In order to gain insight into the likelihood that the collagen scaffold would induce an immune response *in vivo*, the collagen scaffold was initially assessed for immunogenicity *in vitro*, by analysing the expression patterns of pro-inflammatory and anti-inflammatory cytokines.

[00312] It is well known that *in vivo* implantation of suture material results in a level of immune activation, which is deemed clinically acceptable. Therefore, the results of the collagen scaffold were compared to the expression patterns of THP-1 cells exposed to Vicryl® suture material, with expression levels below this control suggesting the collagen scaffold would not cause an adverse response once implanted *in vivo*.

[00313] In this assay, the Vicryl® suture material significantly enhanced the expression of the pro-inflammatory cytokines, such as IL-1 β , TNF- α and IL-8. The collagen scaffold, however, did not increase the expression of these cytokines (Figure 27). The expression of the anti-inflammatory markers was not elevated with any of the tested treatments. These results suggest that the collagen scaffold would not induce an *in vivo* immune response.

Example 31: Results: biomechanics of tendon tissue

[00314] The augmentation of a tendon repair with a biomaterial scaffold should provide the healing tendon with additional mechanical support, to allow the patient to become mobile quicker. Additionally, the function of a tendon is to transfer mechanical forces from the muscle to the bone, and therefore mechanical strength is a measure of its functionality. In this study, the elasticity was tested (the ability of an object or material to resume its normal shape after being stretched) as well as the load to failure (the maximum load applied before the tissue fails). These parameters were tested for the repaired tendons 12 weeks post-surgery.

[00315] It is expected that tendons have a lower elasticity following injury, and tissues with a higher amount of collagen have a higher elasticity. In this study, tendons augmented with the collagen scaffold had a higher elasticity than the non-augmented treatment group, although this did not reach statistical significance (Figure 28). The load to failure of the augmented group was marginally lower than the unaugmented group, although again this was not statistically significant (Figure 28). Overall, these results were consistent with a trend towards improved mechanical support in the augmented group.

Example 31: Results: histology of tendon tissue

[00316] Histological analysis of the repaired tendons allowed evaluation of the structural changes that occur at the tissue level. As part of the evaluation, collagen fibre density was assessed, as well as collagen fibre orientation, the percentage of tendon-bone interface, and also the level of inflammation and vascularisation present. It is noted that both inflammation and vascularisation are contraindicated for tendon healing. These parameters are given a semi-quantitative score by a musculoskeletal pathologist who is blinded to the treatments (Table 5).

[00317] In this assessment, there were no visible differences identified in the structure of the repaired tendons at 12 weeks post-surgery (Figure 29). See also Table 6, below. These results suggest that the collagen scaffold does not adversely affect the level of vascularisation present post-repair, nor does it cause an adverse immune response. It was concluded that the collagen scaffold is a safe and effective material for tendon repair.

Table 6: Histological scoring of tendons augmented with collagen scaffold.

Group	Collagen Fibre Density	Collagen Fibre Orientation	Bone- tendon Interface	Vascularity	Inflammation	Total
Unaugmented	2.950 (± 0.1118)	1.100 (± 0.4541)	1.550 (± 0.3260)	1.100 (± 0.3791)	1.000 (± 0.1768)	7.700 (± 1.1)
Collagen Scaffold Augmentation	2.650 (± 0.4183)	0.8000 (± 0.5420)	1.500 (± 0.1768)	1.300 (± 0.2092)	1.200 (± 0.2092)	7.450 (± 0.412)

References

- [00318] Abercrombie M, Heaysman JEM. Observations on the social behaviour of cells in tissue culture. II. Monolayering of fibroblasts. *Exp Cell Res* 1954 6: 293-306.
- [00319] Adhirajan, N Shanmugasundaram, N Shanmuganathan, S Babu, M. Collagen-based wound dressing for doxycycline delivery: *in-vivo* evaluation in an infected excisional wound model in rats. *J Pharm Pharmacol* 2009a 61: 1617-1623.
- [00320] Adhirajan, N Shanmugasundaram, N Shanmuganathan, S Babu, M. Functionally modified gelatin microspheres impregnated collagen scaffold as novel wound dressing to attenuate the proteases and bacterial growth. *Eur J Pharm Sci* 2009b 36: 235-245.
- [00321] Ahearne M, Yang Y, Then KY, Liu KK. Non-destructive mechanical characterisation of UVA/riboflavin crosslinked collagen hydrogels. *Br J Ophthalmol* 2008 92: 268-71.
- [00322] Akbal, C Lee, SD Packer, SC Davis, MM Rink, RC Kaefer, M. Bladder augmentation with acellular dermal biomatrix in a diseased animal model. *J Urol* 2006 176: 1706-1711.
- [00323] Alekseeva T, Hadjipanayi E, Abou Neel EA, Brown RA. Engineering stable topography in dense bio-mimetic 3D collagen scaffolds. *Eur Cell Mater* 2012 23: 28-40.
- [00324] Altman, AM Chiu, ES Bai, X Yan, Y Song, YH Newsome, RE Alt, EU. Human adipose-derived stem cells adhere to acellular dermal matrix. *Aesthetic Plast Surg* 2008a 32: 698-699.
- [00325] Altman, AM Matthias, N Yan, Y Song, YH Bai, X Chiu, ES Slakey, DP Alt, EU. Dermal matrix as a carrier for *in vivo* delivery of human adipose-derived stem cells. *Biomaterials* 2008b 29: 1431-1442.

- [00326] Andresen LJ, Ledet T, Ehlers N. Keratocyte migration and peptide growth factors: The effect of PDGF, bFGF, EGF, IGF-I, aFGF and TGF- β on human keratocyte migration in a collagen gel. *Curr Eye Res* 1997 16: 605-13.
- [00327] Antonicelli, F Bellon, G Lorimier, S Hornebeck, W. Role of the elastin receptor complex (S-Gal/Cath-A/Neu-1) in skin repair and regeneration. *Wound Repair Regen* 2009 17: 631-638.
- [00328] Ansaloni, L Catena, F.; Gagliardi, S.; Gazzotti, F.; D'Alessandro, L.; Pinna, A.D. Hernia repair with porcine small-intestinal submucosa. *Hernia* 2007 11: 321-326.
- [00329] Archibald, SJ Krarup, C Shefner, J Li, ST Madison, RD. A collagen-based nerve guide conduit for peripheral nerve repair: An electrophysiological study of nerve regeneration in rodents and nonhuman primates. *J Comp Neurol* 1991 306: 685-696.
- [00330] Arffa, RC. 1997. *Grayson's Diseases of the Cornea*. Fourth edition. St. Louis: C. V. Mosby.
- [00331] Arora PD, Narani N, McCulloch CAG. The compliance of collagen gels regulates transforming growth factor- β induction of α -smooth muscle actin in fibroblasts. *Am J Pathol* 1999 154: 871-82.
- [00332] Atala, A Bauer, S Soker, S Yoo, JJ Retik, AB. Tissue-engineered autologous bladders for patients needing cystoplasty. *Lancet* 2006 367: 1241-1246.
- [00333] Avila, MY and JL Navia 2010. Effect of genipin collagen crosslinking on porcine corneas. *J Cataract Refract Surg* 36(4): 659-664.
- [00334] Barbani et al. 1995. Bioartificial materials based on collagen: 1. Collagen cross-linking with gaseous glutaraldehyde. *J Biomater Sci Polym Ed* 7(6): 461-469.
- [00335] Bechetoille, N Dezutter-Dambuyant, C Damour, O Andre, V Orly, I Perrier, E. Effects of solar ultraviolet radiation on engineered human skin equivalent containing both Langerhans cells and dermal dendritic cells. *Tissue Eng* 2007 13: 2667-2679.
- [00336] Berillo D et al. Oxidized dextran as crosslinker for chitosan cryogel scaffolds and formation of polyelectrolyte complexes between chitosan and gelatin, *Macromolecular Bioscience* 12(8): 1090-1099.
- [00337] Blais, M Grenier, M Berthod, F. Improvement of nerve regeneration in tissue-engineered skin enriched with Schwann cells. *J Invest Dermatol* 2009 129: 2895-2900.
- [00338] Beales MP, Funderburgh JL, Jester JV, Hassell JR. Proteoglycan synthesis by bovine keratocytes and corneal fibroblasts: Maintenance of the keratocyte phenotype in culture. *Invest Ophthalmol Vis Sci* 1999 40: 1658-63.

- [00339] Bechtel, JF Muller-Steinhardt, M Schmidtke, C Brunswik, A Stierle, U Sievers, HH. Evaluation of the decellularized pulmonary valve homograft (SynerGraft). *J Heart Valve Dis* 2003 12: 734-740.
- [00340] Beems EM, Van Best JA. Light transmission of the cornea in whole human eyes. *Exp Eye Res* 1990 50:393-5.
- [00341] Belamie E, Mosser G, Gobeaux F, Giraud-Guille MM. Possible transient liquid crystal phase during the laying out of connective tissues: α -chitin and collagen as models. *Journal of Physics: Condensed Matter* 2006 18: S115-S29.
- [00342] Bellows, CF Jian, W McHale, MK Cardenas, D West, JL Lerner, SP Amiel, GE. Blood vessel matrix: A new alternative for abdominal wall reconstruction. *Hernia* 2008 12: 351-358.
- [00343] Bentkover, SH. The biology of facial fillers. *Facial Plast Surg* 2009 25: 73-85.
- [00344] Berthod, F Saintigny, G Chretien, F Hayek, D Collombel, C Damour, O. Optimization of thickness, pore size and mechanical properties of a biomaterial designed for deep burn coverage. *Clin Mater* 1994 15:259-265.
- [00345] Berthod, F Hayek, D Damour, O Collombel, C. Collagen synthesis by fibroblasts cultured within a collagen sponge. *Biomaterials* 1993 14: 749-754.
- [00346] Bidaut-Garnier M, Monnet E, Prongué A, et al. Evolution of corneal graft survival over a 30-year period and comparison of surgical techniques: A cohort study. *Am J Ophthalmol* 2016 163: 59-69.
- [00347] Birk DE, Fitch JM, Linsenmayer TF. Organization of collagen types I and V in the embryonic chicken cornea. *Investigative Ophthalmology & Visual Science* 1986 27: 1470-7.
- [00348] Blais, M Grenier, M Berthod, F. Improvement of nerve regeneration in tissue-engineered skin enriched with Schwann cells. *J Invest Dermatol* 2009 129: 2895-2900.
- [00349] Bouhout, S Perron, E Gauvin, R Bernard, G Ouellet, G Cattani, V Bolduc, S. *In vitro* reconstruction of an autologous, watertight and resistant vesical equivalent. *Tissue Eng A* 2010 16(5): 1539-48.
- [00350] Borene ML, Barocas VH, Hubel A. Mechanical and cellular changes during compaction of a collagen-sponge-based corneal stromal equivalent. *Ann Biomed Eng* 2004 32: 274-83.
- [00351] Bozkurt, A Brook, GA Moellers, S Lassner, F Sellhaus, B Weis, J Woeltje, M Tank, J Beckmann, C Fuchs, P Damink, LO Schugner, F Heschel, I Pallua, N. *In vitro* assessment of axonal growth using dorsal root ganglia explants in a novel three-dimensional collagen matrix. *Tissue Eng* 2007 13: 2971-2979.

- [00352] Bozkurt, A Deumens, R Beckmann, C Olde Damink, L Schugner, F Heschel, I Sellhaus, B Weis, J Jahnen-Dechent, W Brook, GA Pallua, N. *In vitro* cell alignment obtained with a Schwann cell enriched microstructured nerve guide with longitudinal guidance channels. *Biomaterials* 2009 30: 169-179.
- [00353] Brown RA, Wiseman M, Chuo C-B, Cheema U, Nazhat SN. Ultrarapid engineering of biomimetic materials and tissues: Fabrication of nano- and microstructures by plastic compression. *Adv Funct Mater.* 2005 15: 1762-70.
- [00354] Builles N, Janin-Manificat H, Malbouyres M, et al. Use of magnetically oriented orthogonal collagen scaffolds for hemi-corneal reconstruction and regeneration. *Biomaterials* 2010 31: 8313-22.
- [00355] Builles N, Bechetoille N, Justin V, André V, Burillon C, Damour O. Variations in the characteristics of keratocytes in culture in relation to their location in human cornea. *Biomed Mater Eng* 2008 18: 87-98.
- [00356] Carlsson DJ, Li F, Shimmura S, Griffith M. Bioengineered corneas: how close are we? *Curr Opin Ophthalmol* 2003 14: 192-7.
- [00357] Caissie, R Gingras, M Champigny, MF Berthod, F. *In vivo* enhancement of sensory perception recovery in a tissue-engineered skin enriched with laminin. *Biomaterials* 2006 27: 2988-2993.
- [00358] Carrier, P Deschambeault, A Talbot, M Giasson, CJ Auger, FA Guerin, SL Germain, L. Characterization of wound reepithelialization using a new human tissue-engineered corneal wound healing model. *Invest Ophthalmol Vis Sci* 2008 49: 1376-1385.
- [00359] Castoro JA, Bettelheim AA, Bettelheim FA. Water gradients across bovine cornea. *Investigative Ophthalmology & Visual Science* 1988 29: 963-8.
- [00360] Chamberlain, LJ Yannas, IV Hsu, HP Strichartz, G Spector, M. Collagen-GAG substrate enhances the quality of nerve regeneration through collagen tubes up to level of autograft. *Exp Neurol* 1998 154: 315-329.
- [00361] Che, ZM Jung, TH Choi, JH Yoon do, J Jeong, HJ Lee, EJ Kim, J. Collagen-based co-culture for invasive study on cancer cells-fibroblasts interaction. *Biochem Biophys Res Commun* 2006 346: 268-275.
- [00362] Chen, BS Zhang, SL Geng, H Pan, J Chen, F. *Ex vivo* functional evaluation of isolated strips in BAMG tissue-engineered bladders. *Int J Artif Organs* 2009 32: 159-165.
- [00363] Cheuk, M. L., N. Lippok, et al. 2014. Optical coherence tomography imaging of cardiac trabeculae. *Engineering in Medicine and Biology Society (EMBC), 2014 36th Annual International Conference of the IEEE.*

- [00364] Ciardelli, G Gentile, P Chiono, V Mattioli-Belmonte, M Vozzi, G Barbani, N Giusti, P. Enzymatically crosslinked porous composite matrices for bone tissue regeneration. J Biomed Mater Res A 2009 92: 137-151.
- [00365] Cockerill M, Rigozzi MK, Terentjev EM. Mechanosensitivity of the 2nd kind: TGF- β mechanism of cell sensing the substrate stiffness. PLoS One 2015 10: e0139959.
- [00366] Colin, W Donoff, RB. Nerve regeneration through collagen tubes. J Dent Res 1984 63: 987-993.
- [00367] Cook, JL Fox, DB Malaviya, P Tomlinson, JL Farr, J Kuroki, K Cook, CR. Evaluation of small intestinal submucosa grafts for meniscal regeneration in a clinically relevant posterior meniscectomy model in dogs. J Knee Surg 2006 19: 159-167.
- [00368] Corriveau, MP Boufaied, I Lessard, J Chabaud, S Senecal, JL Grodzicky, T Chartier, S Raymond, Y Moulin, VJ. The fibrotic phenotype of systemic sclerosis fibroblasts varies with disease duration and severity of skin involvement: Reconstitution of skin fibrosis development using a tissue engineering approach. J. Pathol 2009 217: 534-542.
- [00369] Cortial, D Gouttenoire, J Rousseau, CF Ronziere, MC Piccardi, N Msika, P Herbage, D Mallein-Gerin, F Freyria, AM. Activation by IL-1 of bovine articular chondrocytes in culture within a 3D collagen-based scaffold. An *in vitro* model to address the effect of compounds with therapeutic potential in osteoarthritis. Osteoarthritis Cartilage 2006 14: 631-640.
- [00370] De Franceschi, L Grigolo, B Roseti, L Facchini, A Fini, M Giavaresi, G Tschon, M Giardino, R. Transplantation of chondrocytes seeded on collagen-based scaffold in cartilage defects in rabbits. J Biomed Mater Res A 2005 75: 612-622.
- [00371] Denner J, Tonjes RR. Infection barriers to successful xenotransplantation focusing on porcine endogenous retroviruses. Clin Microbiol Rev 2012 25: 318-43.
- [00372] Denniston AKO, Murray PI. 2009. Oxford Handbook of Ophthalmology (OUP). Second edition. Oxford: New York. Oxford University Press.
- [00373] Diao J-M, Pang X, Qiu Y, Miao Y, Yu M-M, Fan T-J. Construction of a human corneal stromal equivalent with non-transfected human corneal stromal cells and acellular porcine corneal stromata. Exp Eye Res 2015 132: 216-24.
- [00374] Doillon, CJ Silver, FH. Collagen-based wound dressing: Effects of hyaluronic acid and fibronectin on wound healing. Biomaterials 1986 7: 3-8.
- [00375] Doughty MJ. Changes in hydration, protein and proteoglycan composition of the collagen-keratocyte matrix of the bovine corneal stroma *ex vivo* in a bicarbonate-mixed salts solution, compared to other solutions. Biochim Biophys Acta 2001 1525: 97-107.

- [00376] Dravida, S Gaddipati, S Griffith, M Merrett, K Lakshmi Madhira, S Sangwan, VS Vemuganti, GK. A biomimetic scaffold for culturing limbal stem cells: A promising alternative for clinical transplantation. *J Tissue Eng Regen Med* 2008 2: 263-271.
- [00377] Dreier B, Thomasy SM, Mendonsa R, Raghunathan VK, Russell P, Murphy CJ. Substratum compliance modulates corneal fibroblast to myofibroblast transformation. *Invest Ophthalmol Vis Sci* 2013 54: 5901-7.
- [00378] Du, C Cui, FZ Zhang, W Feng, QL Zhu, XD de Groot, K. Formation of calcium phosphate/collagen composites through mineralization of collagen matrix. *J Biomed Mater Res* 2000 50: 518-527.
- [00379] Dubey, DK Tomar, V. Role of the nanoscale interfacial arrangement in mechanical strength of tropocollagen-hydroxyapatite-based hard biomaterials. *Acta Biomater* 2009 5: 2704-2716.
- [00380] Edmonds, M. Apligraf in the treatment of neuropathic diabetic foot ulcers. *Int J Low Extrem Wounds* 2009 8: 11-18.
- [00381] Eitan, Y Sarig, U Dahan, N Machluf, M. Acellular cardiac extracellular matrix as a scaffold for tissue engineering: *In-vitro* cell support, remodeling and biocompatibility. *Tissue Eng C Methods* 2010 16(4): 671-83.
- [00382] Ekser B, Ezzelarab M, Hara H, et al. Clinical xenotransplantation: The next medical revolution? *Lancet* 2012 379: 672-83.
- [00383] el-Kassaby, A AbouShwareb, T Atala, A. Randomized comparative study between buccal mucosal and acellular bladder matrix grafts in complex anterior urethral strictures. *J Urol* 2008 179: 1432-1436.
- [00384] Ellis, DA Segall, L. Review of non-FDA-approved fillers. *Facial Plast Surg Clin North Am* 2007 15: 239-246.
- [00385] Everaerts, F., M. Torrianni, et al. 2008. Biomechanical properties of carbodiimide crosslinked collagen: influence of the formation of ester crosslinks. *J Biomed Mater Res A* 85(2): 547-555.
- [00386] Eye Bank Association of America. 2013 Eye Banking Statistical Report.
- [00387] Fagerholm P, Lagali NS, Ong JA, et al. Stable corneal regeneration four years after implantation of a cell-free recombinant human collagen scaffold. *Biomaterials* 2014 35:2420-7.
- [00388] Fiala, R Vidlar, A Vrtal, R Belej, K Student, V. Porcine small intestinal submucosa graft for repair of anterior urethral strictures. *Eur Urol* 2007 51: 1702-1708.

- [00389] Farahat, YA Elbahnasy, AM El-Gamal, OM Ramadan, AR El-Abd, SA Taha, MR. Endoscopic urethroplasty using small intestinal submucosal patch in cases of recurrent urethral stricture: A preliminary study. *J Endourol* 2009 23: 2001-2005.
- [00390] Freyria, AM Ronziere, MC Cortial, D Galois, L Hartmann, D Herbage, D Mallein-Gerin, F. Comparative phenotypic analysis of articular chondrocytes cultured within type I or type II collagen scaffolds. *Tissue Eng A* 2009 15: 1233-1245.
- [00391] Funderburgh ML, Mann MM, Funderburgh JL. Keratocyte phenotype is enhanced in the absence of attachment to the substratum. *Mol Vis* 2008 14: 308-17.
- [00392] Funderburgh JL, Funderburgh ML, Mann MM, Corpuz L, Roth MR. Proteoglycan expression during transforming growth factor-induced keratocyte-myofibroblast transdifferentiation. *J Biol Chem* 2001 276: 44173-8.
- [00393] Garcia, Y Hemantkumar, N Collighan, R Griffin, M Rodriguez-Cabello, JC Pandit, A. *In vitro* characterization of a collagen scaffold enzymatically cross-linked with a tailored elastin-like polymer. *Tissue Eng A* 2009 15: 887-899.
- [00394] Gastel, JA Muirhead, WR Lifrak, JT Fadale, PD Hulstyn, MJ Labrador, DP. Meniscal tissue regeneration using a collagenous biomaterial derived from porcine small intestine submucosa. *Arthroscopy* 200117: 151-159.
- [00395] Germain L, Auger FA, Grandbois E, et al. Reconstructed human cornea produced in vitro by tissue engineering. *Pathobiology* 1999 67: 140-7.
- [00396] Ghezzi CE, Rnjak-Kovacina J, Kaplan DL. Corneal tissue engineering: Recent advances and future perspectives. *Tissue Eng Part B Rev* 2015 21:278-87.
- [00397] Glattauer, V White, JF Tsai, WB Tsai, C.C.; Tebb, TA Danon, SJ Werkmeister, JA Ramshaw, JA. Preparation of resorbable collagen-based beads for direct use in tissue engineering and cell therapy applications. *J Biomed Mater Res A* 2010 92: 1301-1309.
- [00398] Girton TS, Dubey N, Tranquillo RT. Magnetic-induced alignment of collagen fibrils in tissue equivalents. *Methods Mol Med* 1999 18: 67-73.
- [00399] Glattauer, V White, JF Tsai, WB Tsai, CC Tebb, TA Danon, SJ Werkmeister, JA Ramshaw, JA. Preparation of resorbable collagen-based beads for direct use in tissue engineering and cell therapy applications. *J Biomed Mater Res A* 2010 92: 1301-1309.
- [00400] Gingras, M Paradis, I Berthod, F. Nerve regeneration in a collagen-chitosan tissue-engineered skin transplanted on nude mice. *Biomaterials* 2003 24: 1653-1661.
- [00401] Gingras, M Beaulieu, MM Gagnon, V Durham, HD Berthod, F. *In vitro* study of axonal migration and myelination of motor neurons in a three-dimensional tissue-engineered model. *Glia* 2008 56: 354-364.

- [00402] Gingras, M Beaulieu, MM Gagnon, V Durham, HD Berthod, F. *In vitro* study of axonal migration and myelination of motor neurons in a three-dimensional tissue-engineered model. *Glia* 2008 56: 354-364.
- [00403] Gomaa A, Comyn O, Liu C. Keratoprostheses in clinical practice - a review. *Clin Experiment Ophthalmol* 2010 38: 211-24.
- [00404] Gonzalez-Andrades M, la Cruz Cardona J de, Ionescu AM, Campos A, del Mar Perez M, Alaminos M. Generation of bioengineered corneas with decellularized xenografts and human keratocytes. *Invest Ophthalmol Vis Sci* 2011 52: 215.
- [00405] Griffith, M Jackson, WB Lagali, N Merrett, K Li, F Fagerholm, P. Artificial corneas: A regenerative medicine approach. *Eye (London)* 2009 23: 1985-1989.
- [00406] Griffith M. Functional human corneal equivalents constructed from cell lines. *Science* 1999 286: 2169-72.
- [00407] Grueterich, M Espana, EM Tseng, SC. *Ex vivo* expansion of limbal epithelial stem cells: Amniotic membrane serving as a stem cell niche. *Surv Ophthalmol* 2003 48: 631-646.
- [00408] Guan L, Ge H, Tang X, et al. Use of a silk fibroin-chitosan scaffold to construct a tissue-engineered corneal stroma. *Cells Tissues Organs* 2013 198:190-7.
- [00409] Guidry C, Grinnell F. Studies on the mechanism of hydrated collagen gel reorganization by human skin fibroblasts. *J Cell Sci* 1985 79:67-81.
- [00410] Guo Q, Phillip JM, Majumdar S, et al. Modulation of keratocyte phenotype by collagen fibril nanoarchitecture in membranes for corneal repair. *Biomaterials* 2013 34: 9365-72.
- [00411] Gurney, TA Kim, DW. Applications of porcine dermal collagen (ENDURAGen) in facial plastic surgery. *Facial Plast Surg Clin North Am* 2007 15: 113-121.
- [00412] Hadjipanayi E, Mudera V, Brown RA. Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness. *J Tissue Eng Regen Med* 2009 3: 77-84.
- [00413] Hara H, Cooper DKC. Xenotransplantation-The future of corneal transplantation? *Cornea*. 2011 30: 371-8.
- [00414] Harley, BA Lynn, AK Wissner-Gross, Z Bonfield, W Yannas, IV Gibson, LJ. Design of a multiphase osteochondral scaffold. II. Fabrication of a mineralized collagen-glycosaminoglycan scaffold. *J Biomed Mater Res A* 2010 92: 1066-1077.
- [00415] Hassanaly SI, Talajic JC, Harissi-Dagher M. Outcomes following Boston type 1 keratoprosthesis implantation in aniridia patients at the University of Montreal. *Am J Ophthalmol* 2014 158: 270-6.

- [00416] Helary C, Bataille I, Abed A, Illoul C, Anglo A, Louedec L, Letourneur D, Meddahi-Pelle A, Giraud-Guille MM. Concentrated collagen hydrogels as dermal substitutes. *Biomaterials* 2010 31: 481-90.
- [00417] Helary C, Foucault-Bertaud A, Godeau G, Coulomb B, Guille MMG. Fibroblast populated dense collagen matrices: cell migration, cell density and metalloproteinases expression. *Biomaterials* 2005 26: 1533-43.
- [00418] <http://www.advancedbiomatrix.com/wp-content/uploads/2013/07/PureCol-Collagen-Gel-Stiffness-and-Rheology-Resutls1.pdf>.
In <http://www.advancedbiomatrix.com/wp-content/uploads/2013/07/PureCol-Collagen-Gel-Stiffness-and-Rheology-Resutls1.pdf>.
- [00419] Hu X, Lui W, Cui L, Wang M, Cao Y. Tissue engineering of nearly transparent corneal stroma. *Tissue Eng* 2005 11: 1710-7.
- [00420] Huang-Lee, LL Wu, JH Nimni, ME. Effects of hyaluronan on collagen fibrillar matrix contraction by fibroblasts. *J Biomed Mater Res* 1994 28: 123-132.
- [00421] Hudson, TW Zawko, S Deister, C Lundy, S Hu, CY Lee, K Schmidt, CE. Optimized acellular nerve graft is immunologically tolerated and supports regeneration. *Tissue Eng* 2004 10: 1641-1651.
- [00422] Hwang YJ, Lyubovitsky JG. Collagen hydrogel characterization: multi-scale and multi-modality approach. *Analytical methods* 2011 3: 529-36.
- [00423] Ignacio Arganda-Carreras, A. C., Verena Kaynig, Johannes Schindelin. 2015. Trainable Weka Segmentation-Fiji. Retrieved from http://fiji.sc/Trainable_Weka_Segmentation.
- [00424] Inoue, T Toda, S Narisawa, Y Sugihara, H. Subcutaneous adipocytes promote the differentiation of squamous cell carcinoma cell line (DJM-1) in collagen gel matrix culture. *J Invest Dermatol* 2001 117: 244-250.
- [00425] Jackson TL. 2008. *Moorfields Manual of Ophthalmology*, Mosby, Elsevier.
- [00426] Jayaraman, M Subramanian, MV. Preparation and characterization of two new composites: Collagen-brushite and collagen octa-calcium phosphate. *Med Sci Monit* 2002 8: BR481-487.
- [00427] Jean, J Lapointe, M Soucy, J Pouliot, R. Development of an *in vitro* psoriatic skin model by tissue engineering. *J Dermatol Sci* 2009 53: 19-25.
- [00428] Jester JV. Corneal crystallins and the development of cellular transparency. *Semin Cell Dev Biol* 2008 19: 82-93.

- [00429] Jester JV, Petroll WM, Barry PA, Cavanagh HD. Expression of alpha-smooth muscle (alpha-sm) actin during corneal stromal wound healing. *Invest Ophthalmol Vis Sci* 1995 36: 809-19.
- [00430] Jester JV, Barry-Lane PA, Cavanagh HD, Petroll WM. Induction of alpha-smooth muscle actin expression and myofibroblast transformation in cultured corneal keratocytes. *Cornea* 1996 15: 505-16.
- [00431] Jester JV, Huang J, Barry-Lane PA, Kao WW-Y, Petroll MW, Cavanagh DH. Transforming growth factor β -mediated corneal myofibroblast differentiation requires actin and fibronectin assembly. *Invest Ophthalmol Vis Sci* 1999 40: 1959-67.
- [00432] Jester JV, Huang J, Matthew Petroll W, Dwight Cavanagh H. TGF β induced myofibroblast differentiation of rabbit keratocytes requires synergistic TGF β , PDGF and integrin signaling. *Exp Eye Res* 2002 75: 645-57.
- [00433] Jester JV, Barry-Lane PA, Petroll WM, Olsen DR, Cavanagh HD. Inhibition of corneal fibrosis by topical application of blocking antibodies to TGF beta in the rabbit. *Cornea* 1997 16: 177-87.
- [00434] Jester JV, Ho-Chang J. Modulation of cultured corneal keratocyte phenotype by growth factors/cytokines control in vitro contractility and extracellular matrix contraction. *Exp Eye Res* 2003 77: 581-92.
- [00435] Jinabhai, A, H Radhakrishnan, C O'Donnell. 2010. Pellucid corneal marginal degeneration: a review. *Contact Lens & Anterior Eye* 34(2): 56-63.
- [00436] Joseph et al. Glucose Sensors in: Wnek GE, Bowlin GL, editors. *Encyclopedia of Biomaterials and Biomedical Engineering*. 2004 New York: Marcel Dekker pp. 683-692.
- [00437] Karr, J. Utilization of living bilayered cell therapy (Apligraf) for heel ulcers. *Adv Skin Wound Care* 2008 21: 270-274.
- [00438] Kaur H, Chaurasia SS, Agrawal V, Suto C, Wilson SE. Corneal myofibroblast viability: Opposing effects of IL-1 and TGF β 1. *Exp Eye Res* 2009 89: 152-8.
- [00439] Kemp, SW Syed, S Walsh, W Zochodne, DW Midha, R. Collagen nerve conduits promote enhanced axonal regeneration, Schwann cell association, and neovascularization compared to silicone conduits. *Tissue Eng A* 2009 15: 1975-1988.
- [00440] Khew, ST Yang, QJ Tong, YW. Enzymatically crosslinked collagen-mimetic dendrimers that promote integrin-targeted cell adhesion. *Biomaterials* 2008 29: 3034-3045.
- [00441] Kim MJ, Yu F, Aldave AJ. Microbial keratitis after Boston type I keratoprosthesis implantation: incidence, organisms, risk factors, and outcomes. *Ophthalmology* 2013 120: 2209-16.

- [00442] Komai Y, Ushiki T. The three-dimensional organization of collagen fibrils in the human cornea and sclera. *Investigative Ophthalmology & Visual Science*. 1991 32: 2244-58.
- [00443] Konuma, T Devaney, EJ Bove, EL Gelehrter, S Hirsch, JC Tavakkol, Z Ohye, RG. Performance of CryoValve SG decellularized pulmonary allografts compared with standard cryopreserved allografts. *Ann Thorac Surg* 2009 88: 849-854.
- [00444] Koob et al. 2001a. Biocompatibility of NDGA-polymerized collagen fibers. I. Evaluation of cytotoxicity with tendon fibroblasts *in vitro*. *J Biomed Mater Res* 56(1): 31-39.
- [00445] Koob et al. 2001b. Biocompatibility of NDGA-polymerized collagen fibers. II. Attachment, proliferation, and migration of tendon fibroblasts *in vitro*. *J Biomed Mater Res* 56(1): 40-48.
- [00446] Koob, T. et al. 2002. Material Properties of polymerized NDGA-collagen composite fibers: development of biologically based tendon constructs. *Biomaterials* 23: 203-212.
- [00447] Koob, T. et al. 2003. Mechanical and thermal properties of novel polymerized NDGA-gelatin hydrogels. *Biomaterials* 24: 1285-1292.
- [00448] Krachmer, JH, Feder, RS, & Belin, MW. 1984. Keratoconus and related noninflammatory corneal thinning disorders. *Survey of Ophthalmology* 28(4): 293-322.
- [00449] Krieg T, Hein R, Hatamochi A, Aumailley M. Molecular and clinical aspects of connective tissue. *Eur J Clin Invest* 1988 18: 105-23.
- [00450] Laflamme, K Roberge, CJ Labonte, J Pouliot, S D'Orleans-Juste, P Auger, FA Germain, L. Tissue-engineered human vascular media with a functional endothelin system. *Circulation* 2005 111: 459-464.
- [00451] Lagali, N Griffith, M Fagerholm, P Merrett, K Huynh, M Munger, R. Innervation of tissue-engineered recombinant human collagen-based corneal substitutes: A comparative *in vivo* confocal microscopy study. *Invest Ophthalmol Vis Sci* 2008 49: 3895-3902.
- [00452] Lakshman N, Petroll WM. Growth factor regulation of corneal keratocyte mechanical phenotypes in 3-D collagen matrices. *Invest Ophthalmol Vis Sci* 2012 53: 1077.
- [00453] Lalan S, Pomerantseva I, Vacanti JP. Tissue engineering and its potential impact on surgery. *World J Surg* 2001 25: 1458-66.
- [00454] Lanfer B, Freudenberg U, Zimmermann R, Stamov D, Korber V, Werner C. Aligned fibrillar collagen matrices obtained by shear flow deposition. *Biomaterials* 2008 29: 3888-95.
- [00455] Lee P, Lin R, Moon J, Lee LP. Microfluidic alignment of collagen fibers for *in vitro* cell culture. *Biomed Microdevices* 2006 8: 35-41.

- [00456] Lekhanont K, Thaweesit P, Muntham D, Chuckpaiwong V, Vongthongsri A. Medium-term outcomes of Boston Type 1 keratoprosthesis implantation in Bangkok, Thailand. *Cornea* 2014 33: 1312-9.
- [00457] Levis HJ, Menzel-Severing J, Drake RAL, Daniels JT. Plastic compressed collagen constructs for ocular cell culture and transplantation: a new and improved technique of confined fluid loss. *Curr Eye Res* 2013 38: 41-52.
- [00458] Levis, H Daniels, JT. New technologies in limbal epithelial stem cell transplantation. *Curr Opin Biotechnol* 2009 20: 593-597.
- [00459] L'Heureux, N Paquet, S Labbe, R Germain, L Auger, FA. A completely biological tissue-engineered human blood vessel. *FASEB J* 1998 12: 47-56.
- [00460] L'Heureux, N Stoclet, JC Auger, FA Lagaud, GJ Germain, L Andriantsitohaina, RA human tissue-engineered vascular media: A new model for pharmacological studies of contractile responses. *FASEB J* 2001 15: 515-524.
- [00461] L'Heureux, N McAllister, TN de la Fuente, LM. Tissue-engineered blood vessel for adult arterial revascularization. *N Engl J Med* 2007 357: 1451-1453.
- [00462] Liao, S Ngiam, M Chan, CK Ramakrishna, S. Fabrication of nano-hydroxyapatite/collagen/osteonectin composites for bone graft applications. *Biomed Mater* 2009 4: 25019.
- [00463] Liu, Y Griffith, M Watsky, MA Forrester, JV Kuffova, L Grant, D Merrett, K Carlsson, DJ. Properties of porcine and recombinant human collagen matrices for optically clear tissue engineering applications. *Biomacromolecules* 2006 7: 1819-1828.
- [00464] Liu, Y Bharadwaj, S Lee, SJ Atala, A Zhang, Y. Optimization of a natural collagen scaffold to aid cell-matrix penetration for urologic tissue engineering. *Biomaterials* 2009 30: 3865-3873.
- [00465] Liyanage, SH Purohit, GS Frye, JN Giordano, P. Anterior abdominal wall reconstruction with a Permacol implant. *J Plast Reconstr Aesthet Surg* 2006 59: 553-555.
- [00466] Long CJ, Roth MR, Tasheva ES, et al. Fibroblast growth factor-2 promotes keratan sulfate proteoglycan expression by keratocytes in vitro. *J Biol Chem* 2000 275: 13918-23.
- [00467] Lynch AP, Ahearne M. Strategies for developing decellularized corneal scaffolds. *Exp Eye Res* 2013 108: 42-7.
- [00468] Magalhaes FP, Hirai FE, de Sousa LB, de Oliveira LA. Boston type 1 keratoprosthesis outcomes in ocular burns. *Acta Ophthalmol (Oxford)* 2013 91: e432-6.

- [00469] Ma, W Fitzgerald, W Liu, QY O'Shaughnessy, TJ Maric, D Lin, HJ Alkon, DL Barker, JL. CNS stem and progenitor cell differentiation into functional neuronal circuits in three-dimensional collagen gels. *Exp Neurol* 2004 190: 276-288.
- [00470] Madaghiele, M Sannino, A Yannas, IV Spector, M. Collagen-based matrices with axially oriented pores. *J Biomed Mater Res A* 2008 85: 757-767.
- [00471] Magnan, M Berthod, F Champigny, MF Soucy, F Bolduc, S. *In vitro* reconstruction of a tissue-engineered endothelialized bladder from a single porcine biopsy. *J Pediatr Urol* 2006 2: 261-270.
- [00472] Mao, J Stosich, MS Moiola, E Lee, CH Fu, S Bastian, B Eisig, S Zemnick, C Ascherman, J Wu, J Rohde, C Ahn, J. Facial Reconstruction by Biosurgery: Cell transplantation vs. cell homing. *Tissue Eng B Rev* 2010 16: 257-262.
- [00473] Marchesi, C et al. Skin-derived stem cells transplanted into resorbable guides provide functional nerve regeneration after sciatic nerve resection. *Glia* 2007 55: 425-438.
- [00474] Martin R, Hulmes D, Farjanel J, Giraud-Guille MM. Molecular liquid crystalline organisation of collagen in physiological conditions. *Biology of the Cell* 1999 91: 276.
- [00475] Matthews JA, Wnek GE, Simpson DG, Bowlin GL. Electrospinning of collagen nanofibers. *Biomacromolecules* 2002 3: 232-8.
- [00476] Maurice DM. The structure and transparency of the cornea. *J Physiol (London)* 1957 136: 263-86.
- [00477] McAllister, TN Maruszewski, M Garrido, SA Wystrychowski, W Dusserre, N Marini, A Zagalski, K Fiorillo, A Avila, H Mangano, X Antonelli, J Kocher, A Zembala, M Cierpka, L de la Fuente, LM L'Heureux, N. Effectiveness of haemodialysis access with an autologous tissue-engineered vascular graft: A multicentre cohort study. *Lancet* 2009 373: 1440-1446.
- [00478] Meek KM, Hayes S. Corneal cross-linking--A review. *Ophthalmic & Physiological Optics: the Journal of the British College of Ophthalmic Opticians*. 2013 33: 78-93.
- [00479] Meshel AS, Wei Q, Adelstein RS, Sheetz MP. Basic mechanism of three-dimensional collagen fibre transport by fibroblasts. *Nat Cell Biol* 2005 7: 157-64.
- [00480] Mi S, Chen B, Wright B, Connon CJ. Plastic compression of a collagen gel forms a much improved scaffold for ocular surface tissue engineering over conventional collagen gels. *J Biomed Mater Res A* 2010 95: 447-53.
- [00481] Mimura T, Amano S, Yokoo S, et al. Tissue engineering of corneal stroma with rabbit fibroblast precursors and gelatin hydrogels. *Mol Vis* 2008 14: 1819-1828.

- [00482] Mimura T, Amano S, Yokoo S, Uchida S, Usui T, Yamagami S. Isolation and distribution of rabbit keratocyte precursors. *Mol Vis* 2008 14: 197-203.
- [00483] Mingin, GC Stock, JA Hanna, MK. Gastrocystoplasty: Long-term complications in 22 patients. *J Urol* 1999 162: 1122-1125.
- [00484] Miron-Mendoza M, Seemann J, Grinnell F. The differential regulation of cell motile activity through matrix stiffness and porosity in three dimensional collagen matrices. *Biomaterials* 2010 31: 6425-35.
- [00485] Mosser G, Anglo A, Helary C, Bouligand Y, Giraud-Guille M-M. Dense tissue-like collagen matrices formed in cell-free conditions. *Matrix Biol* 2006 25: 3-13.
- [00486] Mulder, G Tallis, AJ Marshall, VT Mozingo, D Phillips, L Pierce, GF Chandler, LA Sosnowski, BK. Treatment of nonhealing diabetic foot ulcers with a platelet-derived growth factor gene-activated matrix (GAM501): Results of a phase 1/2 trial. *Wound Repair Regen* 2009 17: 772-779.
- [00487] Müller LJ, Pels L, Vrensen GF. Novel aspects of the ultrastructural organization of human corneal keratocytes. *Invest Ophthalmol Vis Sci* 1995 36: 2557-67.
- [00488] Murphy, CM Haugh, MG O'Brien, FJ. The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering. *Biomaterials* 2010 31: 461-466.
- [00489] Murthy NS. Liquid crystallinity in collagen solutions and magnetic orientation of collagen fibrils. *Biopolymers* 1984 23: 1261-7.
- [00490] Musselmann K, Alexandrou B, Kane B, Hassell JR. Maintenance of the keratocyte phenotype during cell proliferation stimulated by insulin. *J Biol Chem* 2005 280: 32634-9.
- [00491] Musselmann K, Kane BP, Alexandrou B, Hassell JR. IGF-II is present in bovine corneal stroma and activates keratocytes to proliferate in vitro. *Exp Eye Res* 2008 86: 506-11.
- [00492] Myung D, Duhamel PE, Cochran JR, Noolandi J, Ta CN, Frank CW. Development of hydrogel-based keratoprotheses: a materials perspective. *Biotechnol Prog* 2008 24: 735-41.
- [00493] Nie, C Yang, D Morris, SF. Local delivery of adipose-derived stem cells via acellular dermal matrix as a scaffold: A new promising strategy to accelerate wound healing. *Med Hypotheses* 2009 72: 679-682. Tedder, ME Liao, J Weed, B Stabler, C Zhang, H Simionescu, A Simionescu, DT. Stabilized collagen scaffolds for heart valve tissue engineering. *Tissue Eng A* 2009 15: 1257-1268.
- [00494] O'Brien, MF Goldstein, S Walsh, S Black, KS Elkins, R Clarke, D. The SynerGraft valve: A new acellular (nonglutaraldehyde-fixed) tissue heart valve for autologous

recellularization first experimental studies before clinical implantation. *Semin Thorac Cardiovasc Surg* 1999 11: 194-200.

[00495] Oliveira S, R Ringshia, R Legeros, E Clark, L Terracio, C Teixeira, M Yost. An improved collagen scaffold for skeletal regeneration. *Journal of Biomedical Materials* 2009 94(2): 371-379.

[00496] O'Shaughnessy, TJ Lin, HJ Ma, W. Functional synapse formation among rat cortical neurons grown on three-dimensional collagen gels. *Neurosci Lett* 2003 340: 169-172.

[00497] Osborne CS, Barbenel JC, Smith D, Savakis M, Grant MH. Investigation into the tensile properties of collagen/chondroitin-6-sulphate gels: the effect of crosslinking agents and diamines. *Med Biol Eng Comput* 1998 36: 129-34.

[00498] Parenteau-Bareil R, Gauvin R, Berthod, F. Collagen-based biomaterials for tissue engineering applications. *Materials* 2010 3: 31863-1887.

[00499] Parshotam Kumar, G Barker, A Ahmed, S Gerath, J Orford, J. Urinary bladder auto augmentation using INTEGRA((R)) and SURGISIS ((R)): An experimental model. *Pediatr Surg Int* 2010 26: 275-280.

[00500] Patel DV, McKelvie J, Sherwin T, McGhee C. Keratocyte progenitor cell transplantation: A novel therapeutic strategy for corneal disease. *Med Hypotheses* 2013 80:122-4.

[00501] Paul T, Lim M, Starr CE, Lloyd HO, Coleman DJ, Silverman RH. Central corneal thickness measured by the Orbscan II system, contact ultrasound pachymetry, and the Artemis 2 system. *J Cataract Refract Surg.* 2008 34: 1906-12.

[00502] Peters, WJ. Biological dressings in burns-A review. *Ann Plastic Surg* 1980 4: 133–137.

[00503] Petroll WM, Ma L, Kim A, Ly L, Vishwanath M. Dynamic assessment of fibroblast mechanical activity during Rac-induced cell spreading in 3-D culture. *J Cell Physiol* 2008 217: 162-71.

[00504] Pieper, J. S., T. Hafmans, et al. 2000. Development of tailor-made collagen-glycosaminoglycan matrices: EDC/NHS crosslinking, and ultrastructural aspects. *Biomaterials* 21(6): 581-593.

[00505] Pircher M, Gotzinger E, Leitgeb R, Fercher A, Hitzenberger C. Measurement and imaging of water concentration in human cornea with differential absorption optical coherence tomography. *Optics Express* 2003 11: 2190-7.

[00506] Pons-Guiraud, A. Adverse reactions to injectable fillers. *Ann Dermatol Venereol* 2008 135: 171-174.

- [00507] Poole CA, Brookes NH, Clover GM. Confocal imaging of the human keratocyte network using the vital dye 5-chloromethylfluorescein diacetate. *Clin Experiment Ophthalmol* 2003 31: 147-54.
- [00508] Poole CA, Brookes NH, Clover GM. Keratocyte networks visualised in the living cornea using vital dyes. *J Cell Sci* 1993 106: 685-92.
- [00509] Rafat, M., F. Li, et al. 2008. PEG-stabilized carbodiimide crosslinked collagen-chitosan hydrogels for corneal tissue engineering. *Biomaterials* 29(29): 3960-3972.
- [00510] Rafat, M Matsuura, T Li, F Griffith, M. Surface modification of collagen-based artificial cornea for reduced endothelialization. *J Biomed Mater Res A* 2009 88: 755-768.
- [00511] Rabonitz, YS. 2004. Ectatic Disorders of the Cornea. In: *The Cornea*, 4th edition. Lippincott Williams & Wilkins.
- [00512] Rayan GM, Tomasek JJ. Generation of contractile force by cultured Dupuytren's disease and normal palmar fibroblasts. *Tissue Cell* 1994 26: 747-56.
- [00513] Regnier, M Staquet, MJ Schmitt, D Schmidt, R. Integration of Langerhans cells into a pigmented reconstructed human epidermis. *J Invest Dermatol* 1997 109: 510-512.
- [00514] Roy P, Petroll WM, Chuong CJ, Cavanagh HD, Jester JV. Effect of cell migration on the maintenance of tension on a collagen matrix. *Ann Biomed Eng* 1999 27: 721-30.
- [00515] Roycroft A, Mayor R. Molecular basis of contact inhibition of locomotion. *Cell Mol Life Sci* 2015 73: 1119-30.
- [00516] Sabeh, F Shimizu-Hirota, R Weiss, SJ. Protease-dependent versus -independent cancer cell invasion programs: Three-dimensional amoeboid movement revisited. *J Cell Biol* 2009 185: 11-19.
- [00517] Sano, A Maeda, M Nagahara, S Ochiya, T Honma, K Itoh, H Miyata, T Fujioka, K. Atelocollagen for protein and gene delivery. *Adv Drug Deliv Rev* 2003 55: 1651-1677.
- [00518] Schneider, RK Puellen, A Kramann, R Raupach, K Bornemann, J Knuechel, R Perez-Bouza, A Neuss, S. The osteogenic differentiation of adult bone marrow and perinatal umbilical mesenchymal stem cells and matrix remodelling in three-dimensional collagen scaffolds. *Biomaterials* 2010 31: 467-480.
- [00519] Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. *Nat Methods* 2012 9: 671-5.
- [00520] Schwab, IR. Cultured corneal epithelia for ocular surface disease. *Trans. Am Ophthalmol Soc* 1999, 97: 891-986.

- [00521] Shahabeddin, L Berthod, F Damour, O Collombel, C Characterization of skin reconstructed on a chitosan-cross-linked collagen-glycosaminoglycan matrix. *Skin Pharmacol* 1990 3: 107-114.
- [00522] Shanmugasundaram, N Ravichandran, P Reddy, PN Ramamurty, N Pal, S Rao, KP. Collagen-chitosan polymeric scaffolds for the *in vitro* culture of human epidermoid carcinoma cells. *Biomaterials* 2001 22: 1943-1951.
- [00523] Sharkawy et al. Engineering the tissue which encapsulates subcutaneous implants. I. Diffusion properties *J Biomed Mater Res* 1997 37(3): 401-412.
- [00524] Shekarriz, B Upadhyay, J Demirbilek, S Barthold, JS Gonzalez, R. Surgical complications of bladder augmentation: Comparison between various enterocystoplasties in 133 patients. *Urology* 2000 55: 123-128.
- [00525] Sheu et al. 2001. Characterization of collagen gel solutions and collagen matrices for cell culture. *Biomaterials* 22(13): 1713-1719.
- [00526] Shihadeh WA, Mohidat HM. Outcomes of the Boston keratoprosthesis in Jordan. *Middle East African Journal of Ophthalmology*. 2012 19: 97-100.
- [00527] Shortt, AJ Secker, GA Lomas, RJ Wilshaw, SP Kearney, JN Tuft, SJ Daniels, JT. The effect of amniotic membrane preparation method on its ability to serve as a substrate for the *ex-vivo* expansion of limbal epithelial cells. *Biomaterials* 2009 30: 1056-1065.
- [00528] Singh, O, SS Gupta, M Soni, S Moses, S Shukla, RK Mathur. Collagen dressing versus conventional dressings in burn and chronic wounds: a retrospective study. *Journal of Cutaneous and Aesthetic Surgery* 2011 4(1): 12-16.
- [00529] Somers, P Cuvelier, CA Somer, FD Cornelissen, M Cox, E Verloo, M Chiers, K van Nooten, G. Gamma radiation alters the ultrastructure in tissue-engineered heart valve scaffolds. *Tissue Eng. A* 2009 15: 3597-3604.
- [00530] Spencer, NJ Cotanche, DA Klapperich, CM. Peptide- and collagen-based hydrogel substrates for *in vitro* culture of chick cochleae. *Biomaterials* 2008 29: 1028-1042.
- [00531] Sripriya, R Kumar, MS Ahmed, MR Sehgal, PK. Collagen bilayer dressing with ciprofloxacin, an effective system for infected wound healing. *J Biomater Sci Polym Ed* 2007 18: 335-351.
- [00532] Stachowiak, AN Irvine, DJ. Inverse opal hydrogel-collagen composite scaffolds as a supportive microenvironment for immune cell migration. *J Biomed Mater Res A* 2008 85: 815-828.
- [00533] Streeter I, de Leeuw NH. Atomistic modelling of collagen proteins in their fibrillar environment. *Journal of Physical Chemistry B* 2010 114: 13263-70.

- [00534] Sugar A1, Tanner JP, Dontchev M, Tennant B, Schultze RL, Dunn SP, Lindquist TD, Gal RL, Beck RW, Kollman C, Mannis MJ, Holland EJ. Recipient risk factors for graft failure in the cornea donor study. *Ophthalmology*. 2009 116(6): 1023-8.
- [00535] Suh, H Lee, JE. Behavior of fibroblasts on a porous hyaluronic acid incorporated collagen matrix. *Yonsei Med J* 2002 43: 193-202.
- [00536] Sun, W Lin, H Chen, B Zhao, W Zhao, Y Xiao, Z Dai, J. Collagen scaffolds loaded with collagen-binding NGF-beta accelerate ulcer healing. *J Biomed Mater Res A* 2009 92A: 887-895.
- [00537] Sun, W Lin, H Chen, B Zhao, W Zhao, Y Dai, J. Promotion of peripheral nerve growth by collagen scaffolds loaded with collagen-targeting human nerve growth factor-beta. *J Biomed Mater Res A* 2007 83: 1054-1061.
- [00538] Taberner AJ, Han J-C, Loiselle DS, Nielsen PMF. An innovative work-loop calorimeter for *in vitro* measurement of the mechanics and energetics of working cardiac trabeculae.[Erratum appears in *J Appl Physiol* 2012 112(6): 1090 Note: Nielsen, Paul M F [corrected to Nielsen, Poul M F]]. *J Appl Physiol* 2011 111: 1798-803.
- [00539] Takeuchi Y, Fishman J. Long life with or without PERV. *Xenotransplantation* 2010 17: 429-30.
- [00540] Takeshita, F Hokaiwado, N Honma, K Banas, A Ochiya, T. Local and systemic delivery of siRNAs for oligonucleotide therapy. *Methods Mol Biol* 2009 487: 83-92.
- [00541] Takeshita, F Ochiya, T. Therapeutic potential of RNA interference against cancer. *Cancer Sci* 2006 97: 689-696.
- [00542] Tamimi, F Kumarasami, B Doillon, C Gbureck, U Le Nihouannen, D Cabarcos, EL Barralet, JE. Brushite-collagen composites for bone regeneration. *Acta Biomater* 2008 4: 1315-1321.
- [00543] Tan DT, Dart JK, Holland EJ, Kinoshita S. Corneal transplantation. *Lancet* 2012 379: 1749-61.
- [00544] Tebb, TA Tsai, SW Glattauer, V White, JF Ramshaw, JA Werkmeister, JA. Development of porous collagen beads for chondrocyte culture. *Cytotechnology* 2006 52: 99-106.
- [00545] Teebken, OE Puschmann, C Breitenbach, I Rohde, B Burgwitz, K Haverich, A. Preclinical development of tissue-engineered vein valves and venous substitutes using re-endothelialised human vein matrix. *Eur J Vasc Endovasc Surg* 2009 37: 92-102.
- [00546] Teti, A. Regulation of cellular functions by extracellular matrix. *J Am Soc Nephrol* 1992 2: 83-87.

- [00547] Torbet J, Malbouyres M, Builles N, et al. Orthogonal scaffold of magnetically aligned collagen lamellae for corneal stroma reconstruction. *Biomaterials* 2007 28: 4268-76.
- [00548] Tremblay, PL Hudon, V Berthod, F Germain, L Auger, FA. Inosculation of tissue-engineered capillaries with the host's vasculature in a reconstructed skin transplanted on mice. *Am J Transplant* 2005 5: 1002-1010.
- [00549] Trottier, V Marceau-Fortier, G Germain, L Vincent, C Fradette, J. IFATS collection: Using human adipose-derived stem/stromal cells for the production of new skin substitutes. *Stem Cells* 2008 26: 2713-2723.
- [00550] van den Berg TJ, Tan KE. Light transmittance of the human cornea from 320 to 700 nm for different ages. *Vision Research*. 1994 34: 1453-6.
- [00551] Vrana NE, Builles N, Justin V, et al. Development of a reconstructed cornea from collagen-chondroitin sulfate foams and human cell cultures. *Invest Ophthalmol Vis Sci* 2008 49: 5325.
- [00552] Wang HY, Wei RH, Zhao SZ. Evaluation of corneal cell growth on tissue engineering materials as artificial cornea scaffolds. *Int J Ophthalmol* 2013 6:873-8.
- [00553] Williams BR, Gelman RA, Poppe DC, Piez KA. Collagen fibril formation. Optimal *in vitro* conditions and preliminary kinetic results. *J Biol Chem* 1978 253: 6578-85.
- [00554] Williams KA, Lowe M, Bartlett C, Kelly T-L, Coster DJ. Risk factors for human corneal graft failure within the Australian corneal graft registry. *Transplantation* 2008 86: 1720-4.
- [00555] Willshaw H. et al. 2000. *A Handbook of Paediatric Ophthalmology*. Pensord Press: United Kingdom.
- [00556] Wilson SL, Wimpenny I, Ahearne M, Rauz S, Haj AJ El, Yang Y. Chemical and topographical effects on cell differentiation and matrix elasticity in a corneal stromal layer model. *Adv Funct Mater* 2012 22:3641-9.
- [00557] Wolf, K Muller, R Borgmann, S Brocker, EB Friedl, P. Amoeboid shape change and contact guidance: T-lymphocyte crawling through fibrillar collagen is independent of matrix remodeling by MMPs and other proteases. *Blood* 2003 102: 3262-3269.
- [00558] Wollensak G, Aurich H, Pham DT, Wirbelauer C. Hydration behavior of porcine cornea crosslinked with riboflavin and ultraviolet A. *J Cataract Refract Surg*. 2007;33:516-21.
- [00559] Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and porcine corneas after riboflavin-ultraviolet-A-induced cross-linking. *J Cataract Refract Surg* 2003 29 1780-5.

- [00560] Wollensak, G. 2006. Crosslinking treatment of progressive keratoconus: new hope. *Curr Opin Ophthalmol* 17(4): 356-360.
- [00561] Wollensak, G. and E. Iomdina 2009. Long-term biomechanical properties of rabbit cornea after photodynamic collagen crosslinking. *Acta Ophthalmol* 87(1): 48-51.
- [00562] Wu J, Du Y, Mann MM, Funderburgh JL, Wagner WR. Corneal stromal stem cells versus corneal fibroblasts in generating structurally appropriate corneal stromal tissue. *Exp Eye Res* 2014 120:71-81.
- [00563] Wu J, Du Y, Mann MM, Yang E, Funderburgh JL, Wagner WR. Bioengineering organized, multilamellar human corneal stromal tissue by growth factor supplementation on highly aligned synthetic substrates. *Tissue Eng Part A* 2013 19:2063-75.
- [00564] Xu, T Molnar, P Gregory, C Das, M Boland, T Hickman, JJ. Electrophysiological characterization of embryonic hippocampal neurons cultured in a 3D collagen hydrogel. *Biomaterials* 2009 30: 4377-4383.
- [00565] Yamagami S, Yokoo S, Mimura T, Takato T, Araie M, Amano S. Distribution of precursors in human corneal stromal cells and endothelial cells. *Ophthalmology* 2007 114: 433-9.
- [00566] Yanai R, Yamada N, Kugimiya N, Inui M, Nishida T. Mitogenic and antiapoptotic effects of various growth factors on human corneal fibroblasts. *Invest Ophthalmol Vis Sci* 2002 43: 2122-6.
- [00567] Yannas, IV Burke, JF Orgill, DP Skrabut, EM. Wound tissue can utilize a polymeric template to synthesize a functional extension of skin. *Science* 1982 215: 174-176.
- [00568] Yang S, Leong KF, Du Z, Chua CK. The design of scaffolds for use in tissue engineering. Part I. Traditional factors. *Tissue Eng* 2001 7: 679-89.
- [00569] Yeo, TK Brown, L Dvorak, HF Alterations in proteoglycan synthesis common to healing wounds and tumors. *Am J Pathol* 1991 138: 1437-1450.
- [00570] Yeung T, Georges PC, Flanagan LA, et al. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton* 2005 60: 24-34.
- [00571] Yu T, Rajendran V, Griffith M, Forrester JV, Kuffová L. High-risk corneal allografts: A therapeutic challenge. *World J Transplant* 2016 6: 10.
- [00572] Yung, CW Wu, LQ Tullman, JA Payne, GF Bentley, WE Barbari, TA. Transglutaminase crosslinked gelatin as a tissue engineering scaffold. *J Biomed Mater Res A* 2007 83: 1039-1046.

[00573] Zakaria, N Koppen, C Van Tendeloo, V Berneman, Z Hopkinson, A Tassignon, MJ. Standardized limbal epithelial stem cell graft generation and transplantation. *Tissue Eng C Methods* 2010 16(5): 921-7.

[00574] Zhang Q, Lu H, Kawazoe N, Chen G. Preparation of collagen scaffolds with controlled pore structures and improved mechanical property for cartilage tissue engineering. *Journal of Bioactive and Compatible Polymers* 2013 28: 426-438.

[00575] Zhang J, Sisley AMG, Anderson AJ, Taberner AJ, McGhee CNJ, Patel DV. Characterization of a novel collagen scaffold for corneal tissue engineering. *Tissue Eng Part C Methods* 2015 22: 165-172.

[00576] Zhong, S Yung, LY. Enhanced biological stability of collagen with incorporation of PAMAM dendrimer. *J Biomed Mater Res A* 2009 91: 114-122.

[00577] A person of ordinary skill in the art will readily appreciate from the disclosure that later modifications, substitutions, and/or variations performing substantially the same function or achieving substantially the same result as embodiments or aspects described herein may be utilised according to such related embodiments or aspects of the present invention. Thus, the invention is intended to encompass, within its scope, the modifications, substitutions, and variations to processes, manufactures, compositions of matter, compounds, means, methods, and/or steps disclosed herein.

[00578] All references, including patents and patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. Nor does discussion of any reference constitute an admission that such reference forms part of the common general knowledge in the art, in New Zealand or in any other country.

WHAT IS CLAIMED IS:

1. A method of preparing a collagen composition, the method comprising:
 - a) obtaining a collagen gel having a collagen concentration of 100 mg/ml to 190 mg/ml;
 - b) hydrating the collagen gel such that liquid content is increased 10-fold to 20-fold;and
 - c) combining two or more aliquots of hydrated collagen gel such that a combined gel aliquot of increased thickness is obtained.
2. The method according to claim 1, which further comprises: moulding the combined gel aliquot.
3. The method according to claim 1 or claim 2, which further comprises: neutralising the pH of the combined gel aliquot.
4. The method according to any one of claims 1 to 3, which further comprises: heating the combined gel aliquot under humidity, thereby allowing the gel to set.
5. The method according to claim 4, which further comprises: dehydrating the set gel to completion.
6. The method according to claim 5, which further comprises: rehydrating the dehydrated gel by immersion in a liquid.
7. The method according to claim 6, wherein the method further comprises: crosslinking the combined gel aliquot following the dehydrating and the rehydrating steps.
8. The method according to any one of claims 4 to 7, wherein the moulding step is carried out at the same time as the heating step.
9. The method according to any one of claims 4 to 7, wherein the neutralising step is carried out prior to or at the same time as the heating step.

10. The method according to any one of claims 1 to 9, wherein the concentration of (a) is obtained by centrifugation and ultrafiltration of a collagen solution.
11. The method according to any one of claims 1 to 9, wherein the concentration of (a) is obtained by concentrating a collagen solution 10-fold to 50-fold by centrifugation in combination with ultrafiltration.
12. The method according to any one of claims 1 to 11, wherein the hydrating of (b) is carried out by centrifugation of the collagen gel in the presence of the liquid.
13. The method according to any one of claims 1 to 12, wherein the collagen gel is compressed prior to (b).
14. The method according to any one of claims 1 to 13, wherein the combining of (c) is carried out by centrifugation of the two or more aliquots of hydrated collagen gel.
15. The method according to any one of claims 10 to 14, wherein the collagen solution has a collagen concentration of 3 mg/ml to 10 mg/ml, and wherein the collagen solution comprises collagen provided in an acetic acid solution.
16. The method according to any one of claims 10 to 15, wherein the ultrafiltration is carried out with a molecular weight cut-off of 10 kDa.
17. The method according to any one of claims 10 to 16, wherein the centrifugation is carried out at 2000 x g to 4000 x g.
18. The method according to any one of claims 10 to 17, wherein the centrifugation is carried out at 2°C to 8°C.
19. The method according to any one of claims 1 to 18, wherein the liquid is selected from the group consisting of: water, cell culture media, and a solution comprising a therapeutic agent.

20. The method according to any one of claims 1 to 19, wherein the collagen is type I collagen.
21. The method according to claim 20, wherein the type I collagen is bovine collagen, human collagen, or recombinant human collagen.
22. The method according to any one of claims 7 to 21, wherein the crosslinking is carried out by a crosslinking agent selected from the group consisting of genipin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and UVA combined with riboflavin.
23. A method of preparing a collagen composition, the method comprising:
obtaining a collagen gel having a collagen concentration of 100 mg/ml to 190 mg/ml by centrifugation and ultrafiltration of a collagen solution, with the proviso that the collagen solution is substantially free of hydrochloric acid.
24. The method according to claim 23, which further comprises: hydrating the collagen gel such that liquid content is increased 10-fold to 20-fold.
25. The method according to claim 23 or claim 24, which further comprises: compressing the collagen gel.
26. The method according to any one of claims 23 to 25, which further comprises: neutralising the pH of the collagen gel.
27. The method according to any one of claims 23 to 26, wherein the ultrafiltration is carried out with a molecular weight cut-off of 10 kDa.
28. The method according to any one of claims 23 to 27, wherein the centrifugation is carried out at 2000 x g to 4000 x g.
29. The method according to any one of claims 23 to 28, wherein the collagen solution has a collagen concentration of 3 mg/ml to 10 mg/ml, and wherein the collagen solution comprises collagen provided in an acetic acid solution.

30. A collagen composition that comprises: a water content of 78% to 88% w/w, a transmittance of at least 0.85 for wavelengths of 570 nm, 750 nm, and/or 900 nm, a mean ultimate tensile strength of 1 MPa to 1.5 MPa at a strain rate of 0.5% s⁻¹, when uncrosslinked.
31. A collagen composition prepared according to the method of any one of claims 1 to 29.
32. The collagen composition according to claim 31, which comprises a water content of 78% to 88% w/w.
33. The collagen composition according to claim 31 or claim 32, which comprises a transmittance of at least 0.85 for wavelengths of 570 nm, 750 nm, and/or 900 nm.
34. The collagen composition according to any one of claims 31 to 33, which comprises a mean ultimate tensile strength of 1 MPa to 1.5 MPa at a strain rate of 0.5% s⁻¹, when uncrosslinked.
35. The collagen composition according to any one of claims 31 to 33, which comprises a mean ultimate tensile strength of 1.5 MPa to 5.5 MPa at a strain rate of 0.5% s⁻¹, when crosslinked by a crosslinking agent selected from the group consisting of: genipin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and UVA combined with riboflavin.
36. The collagen composition according to any one of claims 31 to 35, which comprises one or more of:
- a) an extracellular matrix component selected from the group consisting of: chitosan, proteoglycans, glycosaminoglycans, elastin, fibronectin, and laminin, and any combination thereof;
 - b) a growth factor selected from the group consisting of: FGF, VEGF, PDGF, EGF, IGF, TGF, HGF, KGF, and BMP growth factors, and any combination thereof;
 - c) a cell selected from the group consisting of: stem cells, progenitor cells, transient amplifying cells, mature cells, and any combination thereof;
 - d) a cell selected from the group consisting of: keratocytes, chondrocytes, osteocytes, tenocytes, neurons, epithelial cells, keratinocytes, and any combination thereof;
 - e) a protease inhibitor selected from the group consisting of: aprotinin, bestatin, pepstatin, tissue inhibitors of metalloproteinases (TIMP), and any combination thereof; and

f) a therapeutic agent selected from the group consisting of: anti-inflammatories, anti-microbials, antihistamines, steroids, sympathomimetics, beta receptor blockers, parasympathomimetics, parasympatholytics, prostaglandins, nutrients, vasoconstrictors, lubricants, anaesthetics, growth factors, and any combination thereof.

37. The collagen composition according to claim 36, wherein the cell is introduced following administration of the collagen composition.

38. The collagen composition according to any one of claims 31 to 37, which is prepared as a corneal tissue substitute, carrier for corneal cells, corneal implant, corneal inlay, corneal patch, or therapeutic contact lens.

39. A kit comprising the collagen composition according to any one of claims 31 to 38.

40. A method of repairing or augmenting tissue of a subject, the method comprising: administering the collagen composition of any one of claims 31 to 38 to a tissue site of the subject, thereby providing repair or augmentation of the tissue.

41. The method according to claim 40, wherein the collagen composition comprises one or more of:

a) an extracellular matrix component selected from the group consisting of: chitosan, proteoglycans, glycosaminoglycans, elastin, fibronectin, and laminin, and any combination thereof;

b) a growth factor selected from the group consisting of: FGF, VEGF, PDGF, EGF, IGF, TGF, HGF, KGF, and BMP growth factors, and any combination thereof;

c) a cell selected from the group consisting of: stem cells, progenitor cells, transient amplifying cells, mature cells, and any combination thereof;

d) a cell selected from the group consisting of: keratocytes, chondrocytes, osteocytes, tenocytes, neurons, epithelial cells, keratinocytes, and any combination thereof;

e) a protease inhibitor selected from the group consisting of: aprotinin, bestatin, pepstatin, tissue inhibitors of metalloproteinases (TIMP), and any combination thereof; and

f) a therapeutic agent selected from the group consisting of: anti-inflammatories, anti-microbials, antihistamines, steroids, sympathomimetics, beta receptor blockers,

parasympathomimetics, parasympatholytics, prostaglandins, nutrients, vasoconstrictors, lubricants, anaesthetics, growth factors, and any combination thereof.

42. The method according to claim 41, wherein the cell is introduced following administration of the collagen composition.

43. The method according to any one of claims 40 to 42, wherein the tissue is an epithelial tissue.

44. The method according to any one of claims 40 to 42, wherein the tissue is skin.

45. The method according to claim 44, wherein the tissue comprises an epidermal, dermal, or hypodermal layer.

46. The method according to any one of claims 40 to 42, wherein the tissue is corneal, retinal, conjunctival, or optic nerve tissue.

47. The method according to claim 46, wherein the tissue comprises a stromal layer.

48. The method according to any one of claims 40 to 42, wherein the tissue is selected from the group consisting of: bone, muscle, nerve, and connective tissue, and any combination thereof.

49. The method according to claim 48, wherein the connective tissue is tendon or cartilage tissue.

50. The method according to any one of claims 40 to 42, wherein the tissue is selected from the group consisting of: chondral, osteochondral, genitourinary, cardiovascular, neuronal tissue, and any combination thereof.

51. The method according to any one of claims 40 to 50, wherein the tissue site is affected by one or more of: tissue thinning, tissue weakening, tissue loss, cell loss, matrix loss, collagen loss, or a defect in tissue form.

52. A method of repairing or augmenting corneal tissue of a subject, the method comprising: administering the collagen composition according to any one of claims 31 to 38 to a corneal tissue site of the subject, thereby providing repair or augmentation of the corneal tissue.

53. The method according to claim 52, wherein the collagen composition comprises one or more of:

a) an extracellular matrix component selected from the group consisting of: chitosan, proteoglycans, glycosaminoglycans, elastin, fibronectin, and laminin, and any combination thereof;

b) a growth factor selected from the group consisting of: EGF, KGF, HGF, IGF, and TGF growth factors, and any combination thereof;

c) a cell selected from the group consisting of: stem cells, progenitor cells, transient amplifying cells, mature cells, and any combination thereof;

d) a cell selected from the group consisting of: keratocytes, epithelial cells, and any combination thereof;

e) a protease inhibitor selected from the group consisting of: aprotinin, bestatin, pepstatin, tissue inhibitors of metalloproteinases (TIMP), and any combination thereof; and

f) a therapeutic agent selected from the group consisting of: anti-inflammatories, anti-microbials, antihistamines, steroids, sympathomimetics, beta receptor blockers, parasympathomimetics, parasympatholytics, prostaglandins, nutrients, vasoconstrictors, lubricants, anaesthetics, growth factors, and any combination thereof.

54. The method according to claim 53, wherein the cell is introduced following administration of the collagen composition.

55. The method according to any one of claims 52 to 54, wherein the tissue comprises a stromal layer.

56. The method according to any one of claims 52 to 55, wherein the collagen composition is prepared as a corneal tissue substitute, carrier for corneal cells, corneal implant, corneal inlay, corneal patch, or therapeutic contact lens.

57. The method according to any one of claims 52 to 56, wherein the corneal tissue site is affected by one or more of the group consisting of: tissue thinning, tissue weakening, cell loss, tissue loss, matrix loss, collagen loss, and tissue irregularity.
58. The method according to any one of claims 52 to 56, wherein the subject is affected by a condition selected from the group consisting of: corneal ectasias, corneal dystrophies, corneal ulcers, corneal infections, corneal wounds, and any combination thereof.
59. The method according to claim 58, wherein the corneal wound is caused by physical injury, chemical injury, radiation damage, and/or damage from medication.
60. The method according to claim 58, wherein the corneal wound is a surgical wound.

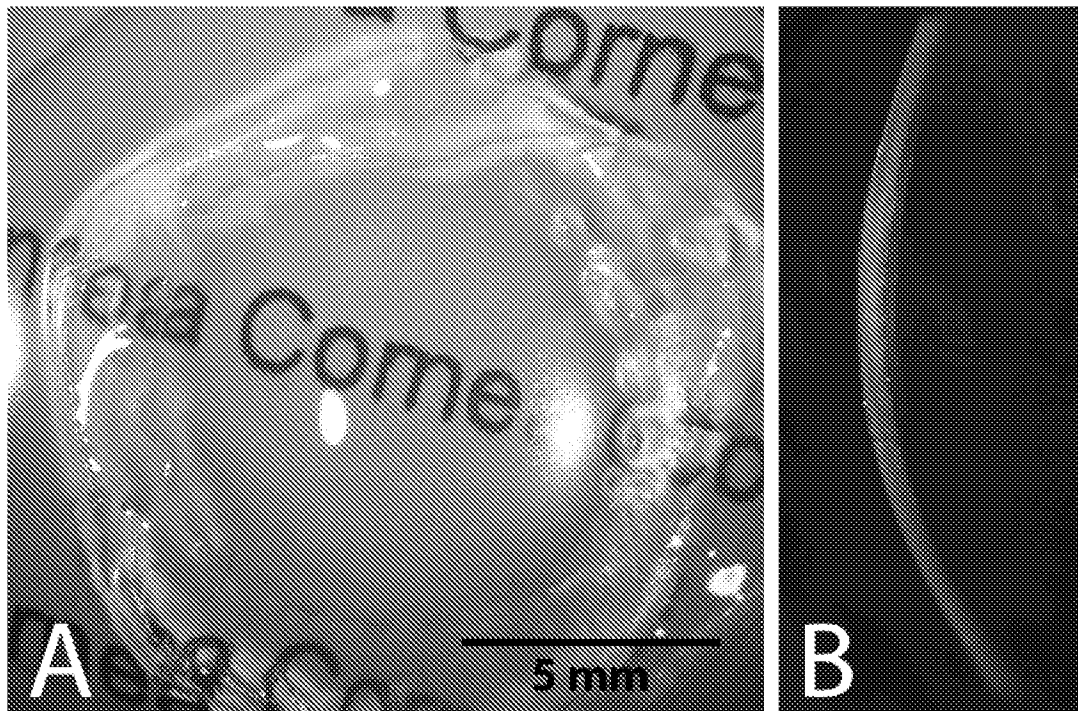
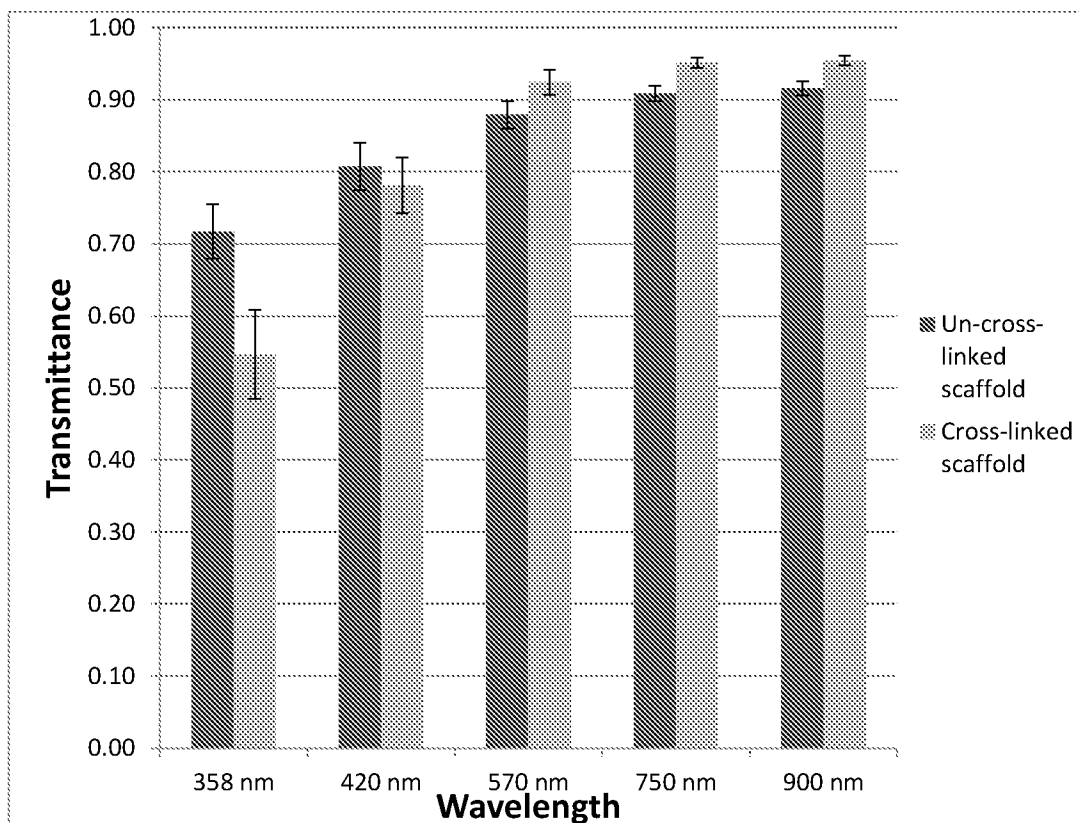
Figure 1**Figure 2**

Figure 3

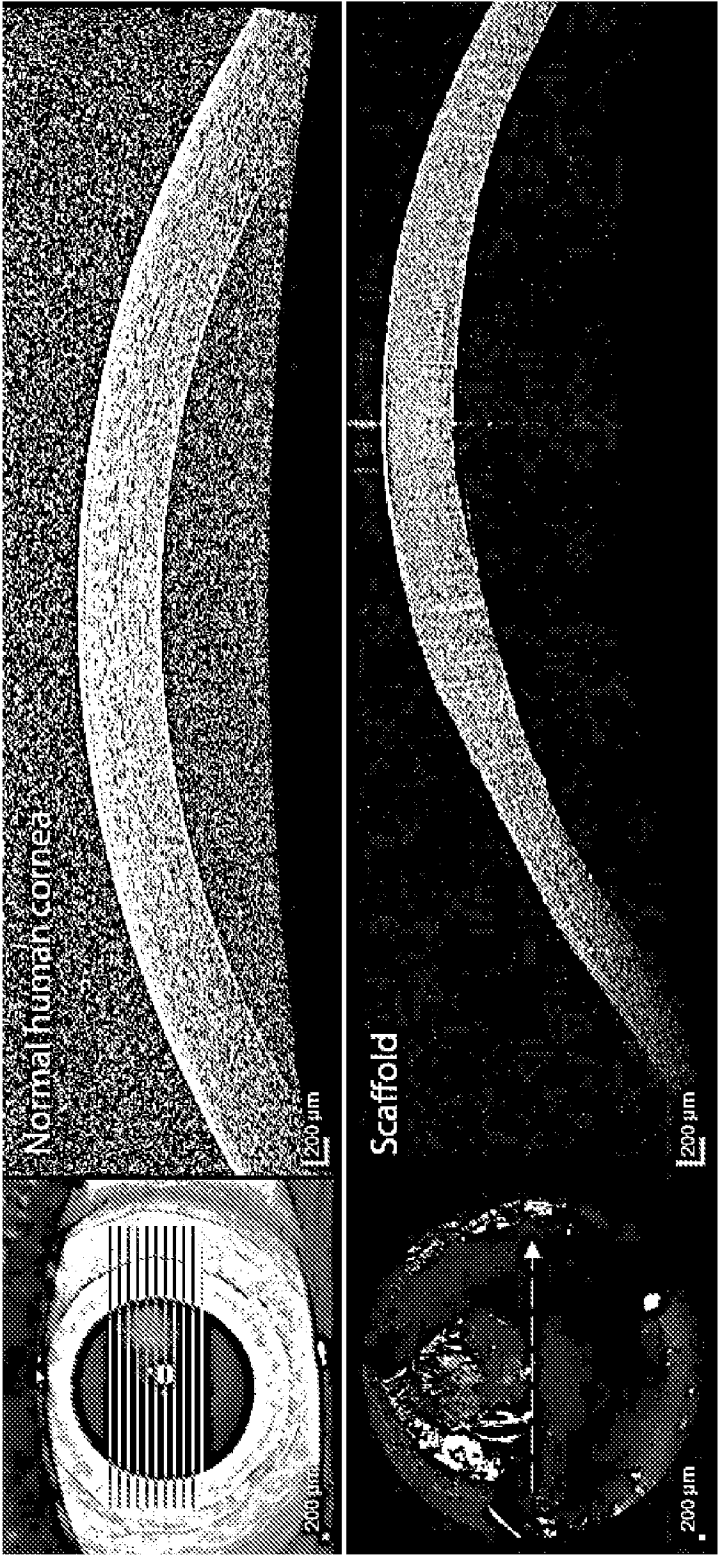


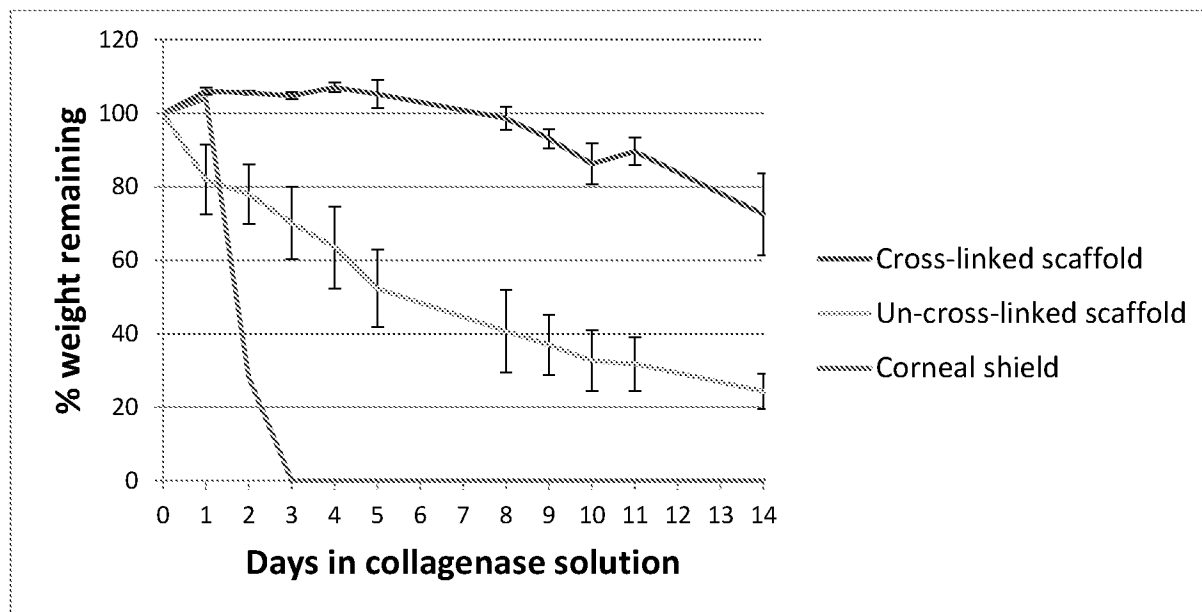
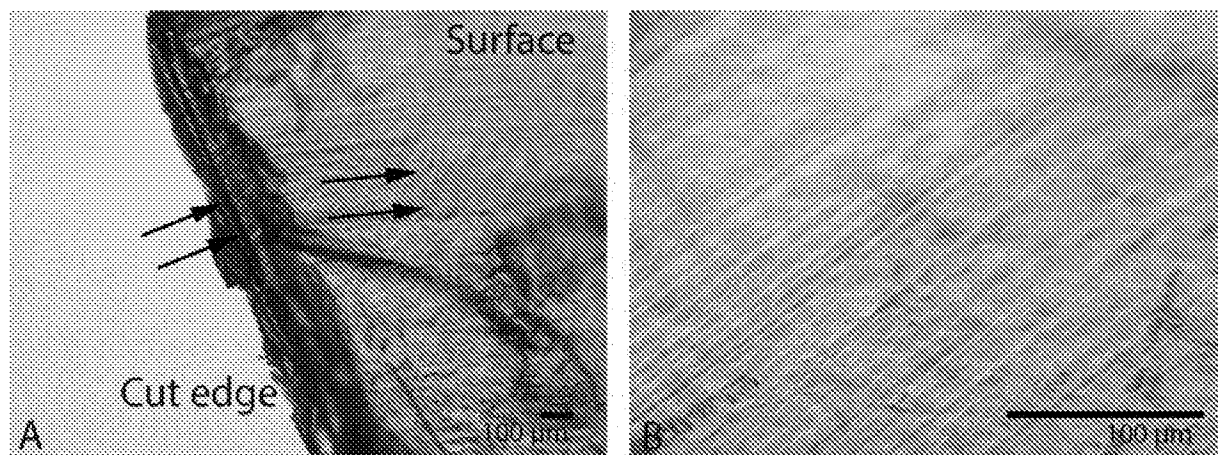
Figure 4**Figure 5**

Figure 6

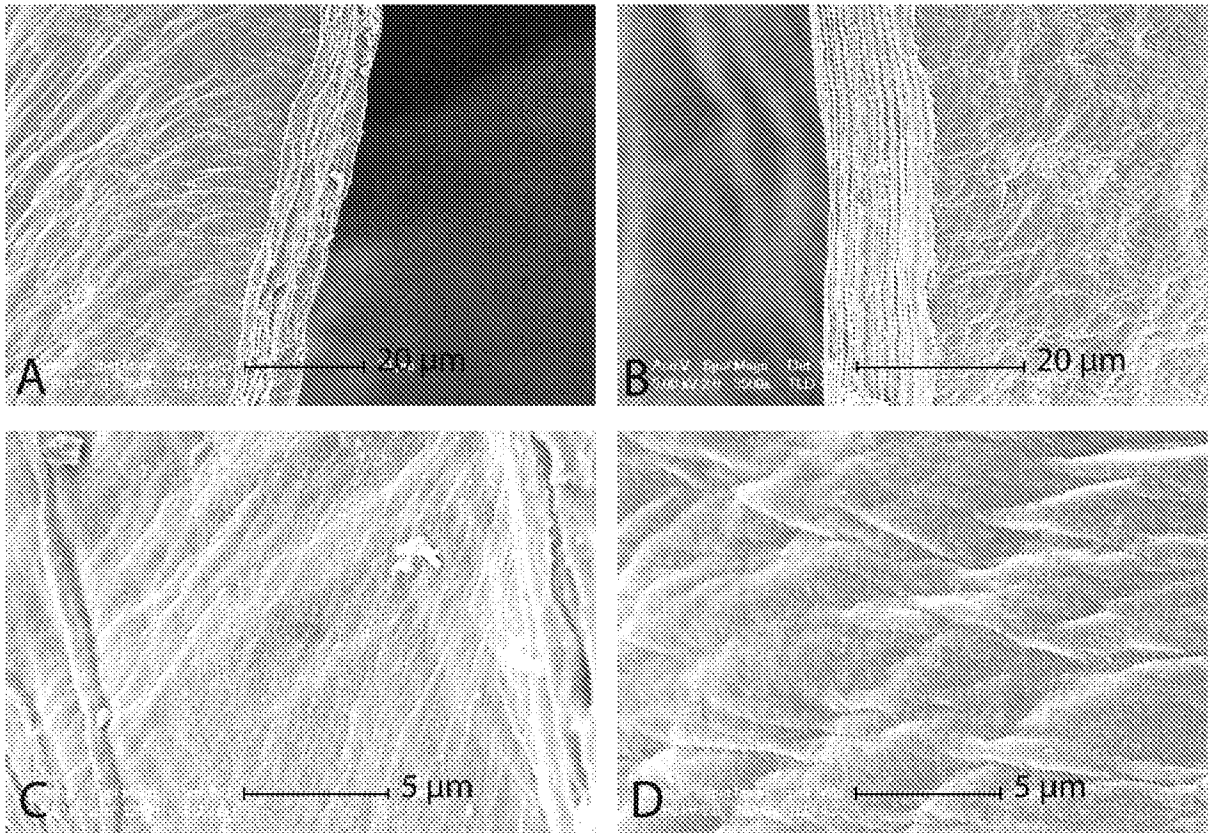


Figure 7

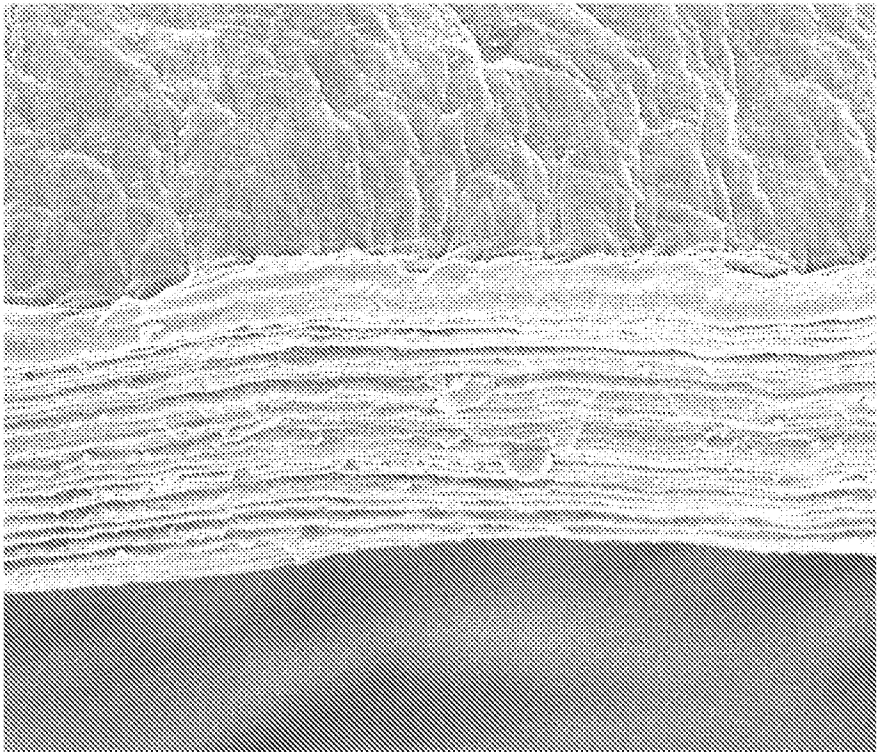


Figure 8

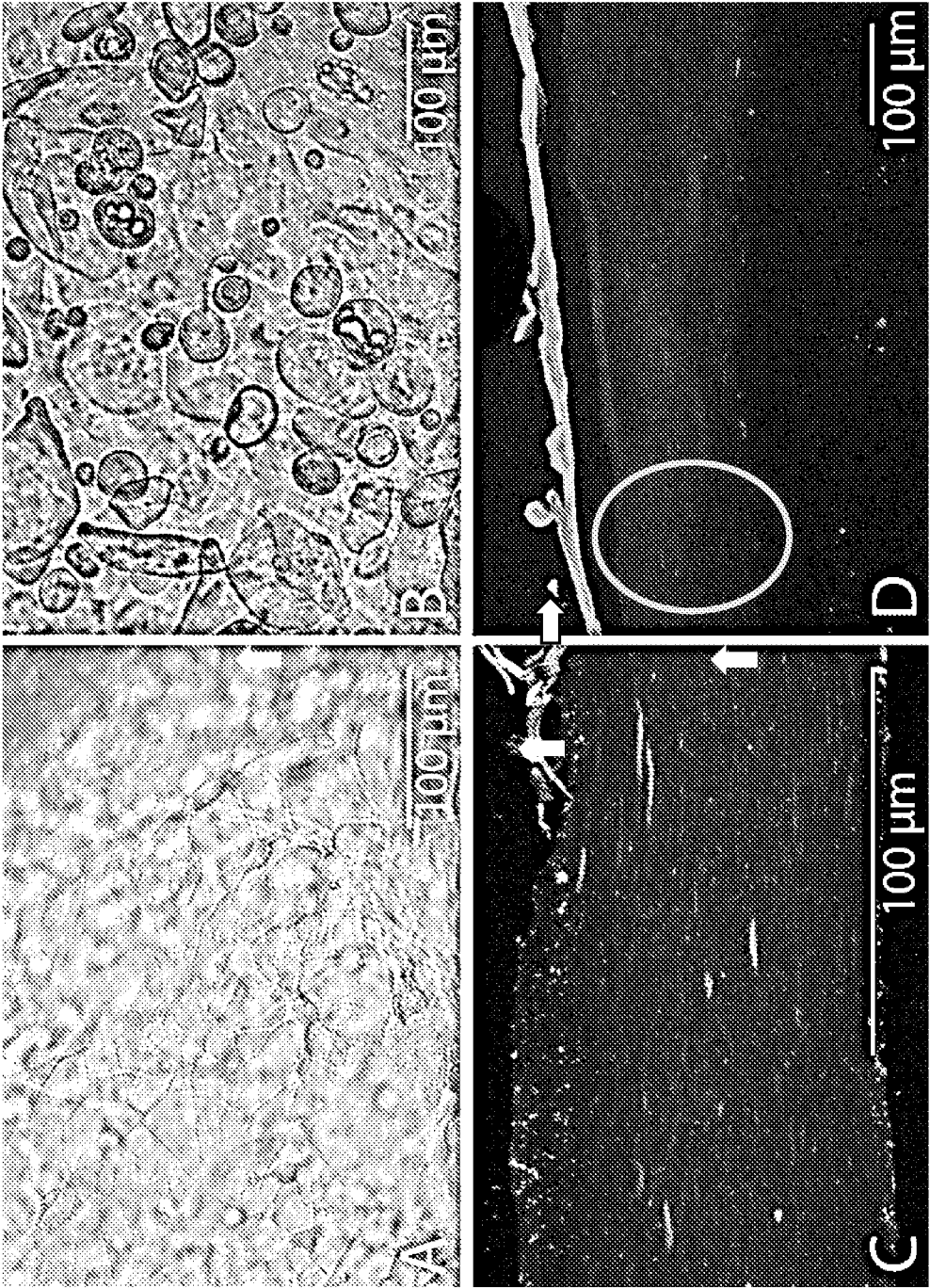


Figure 9

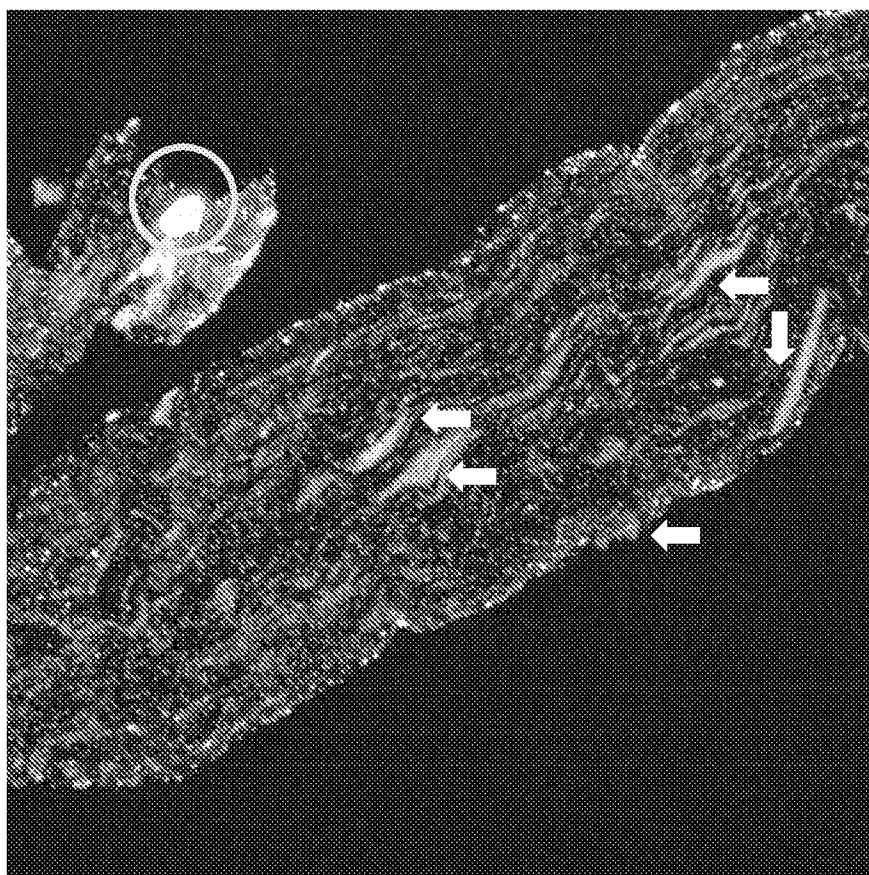
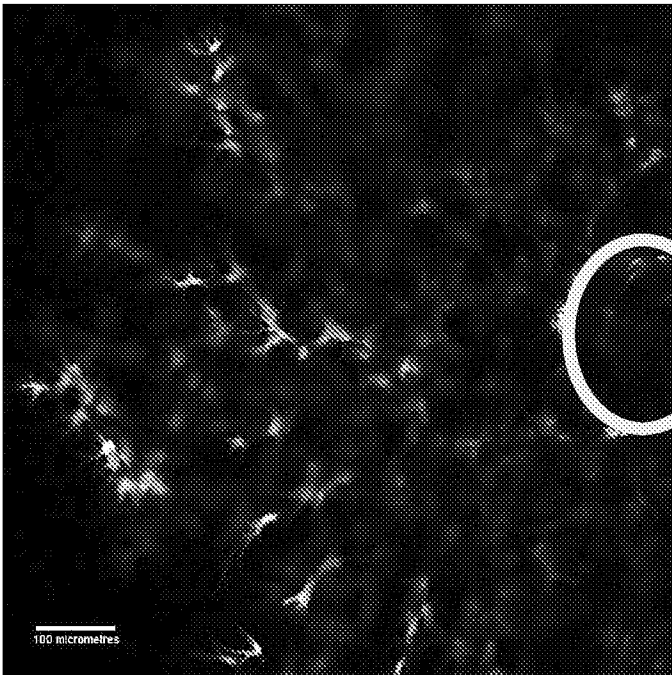
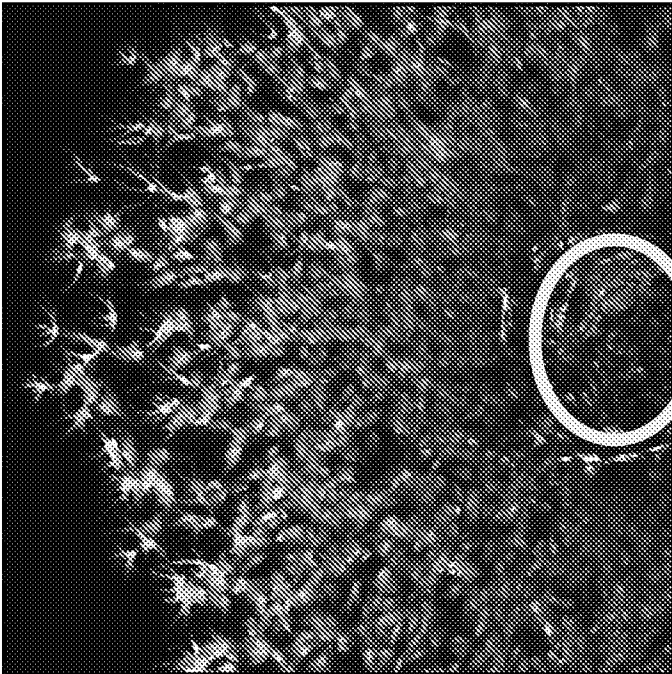


Figure 10

A



B



C

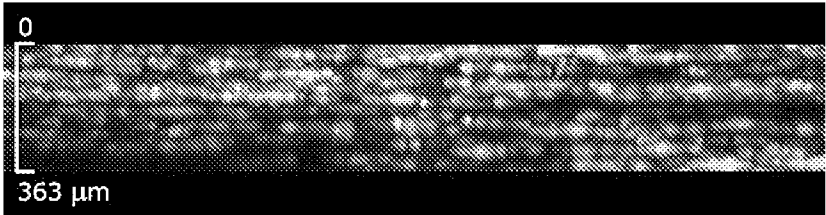


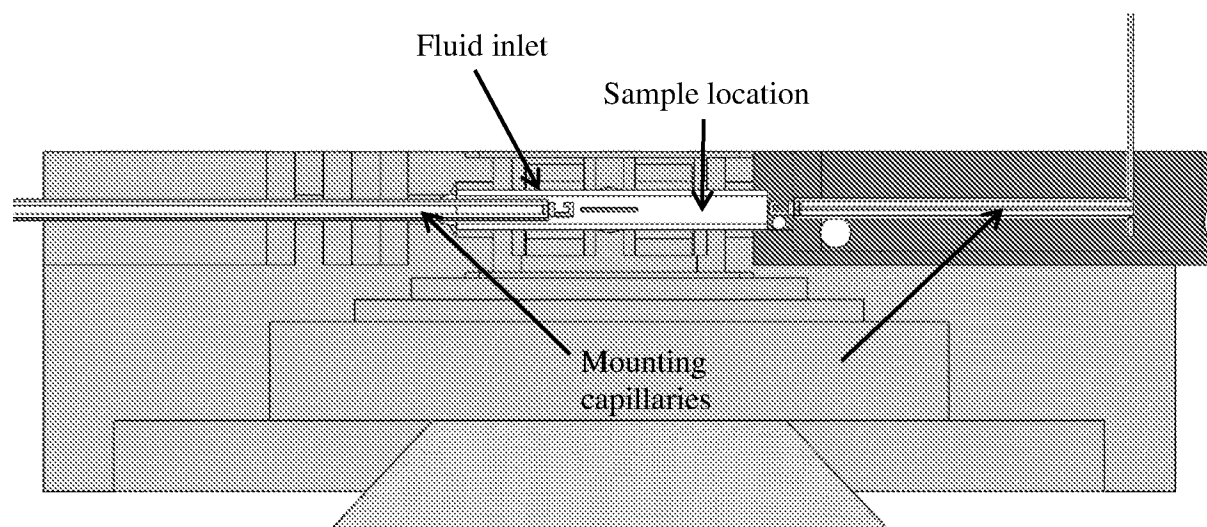
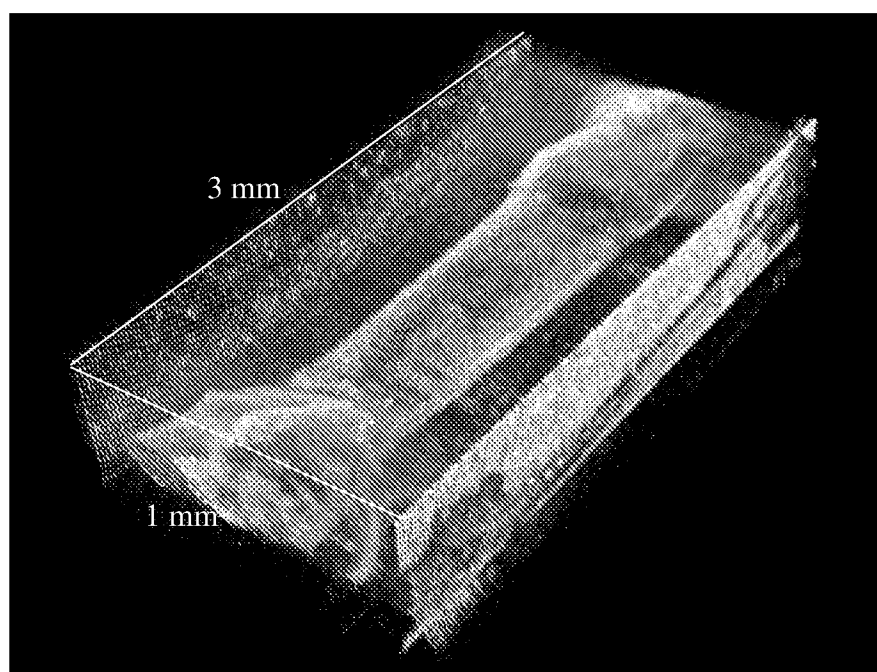
Figure 11**Figure 12**

Figure 13

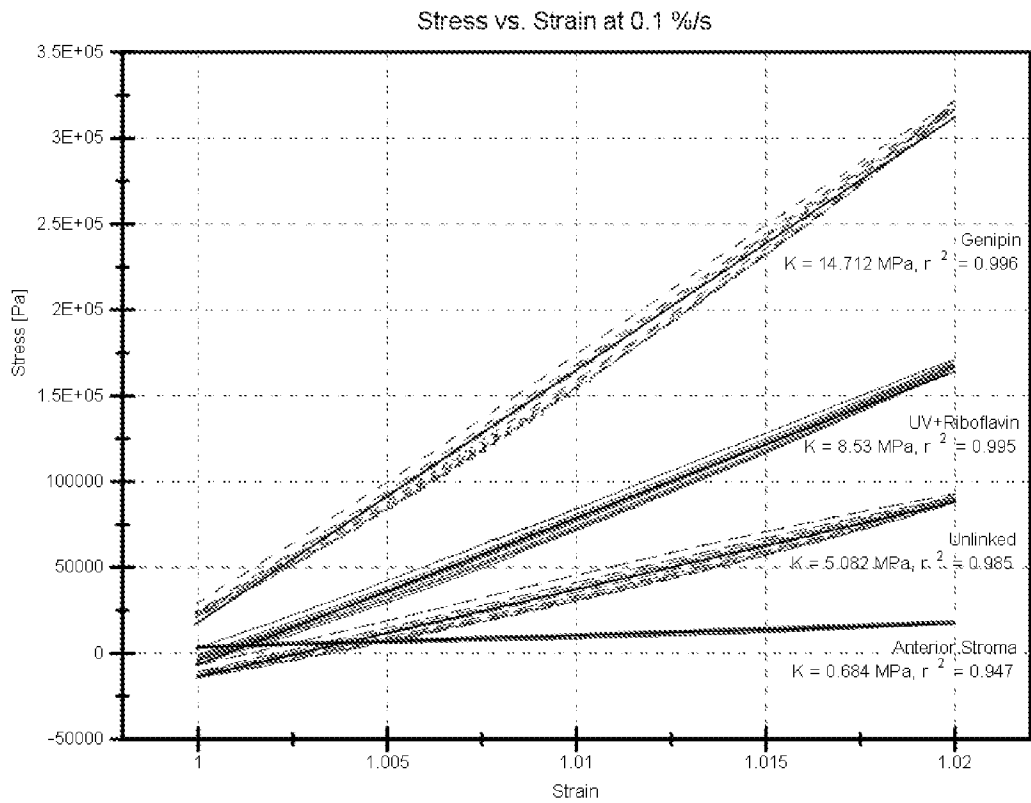


Figure 14

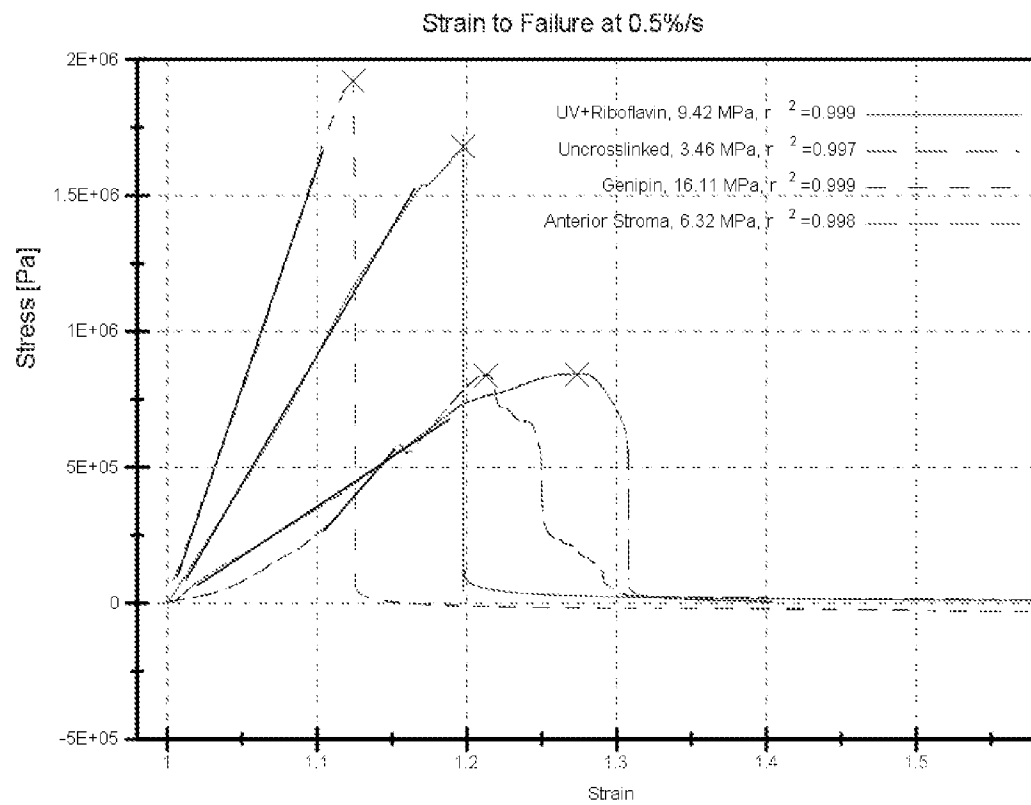


Figure 15

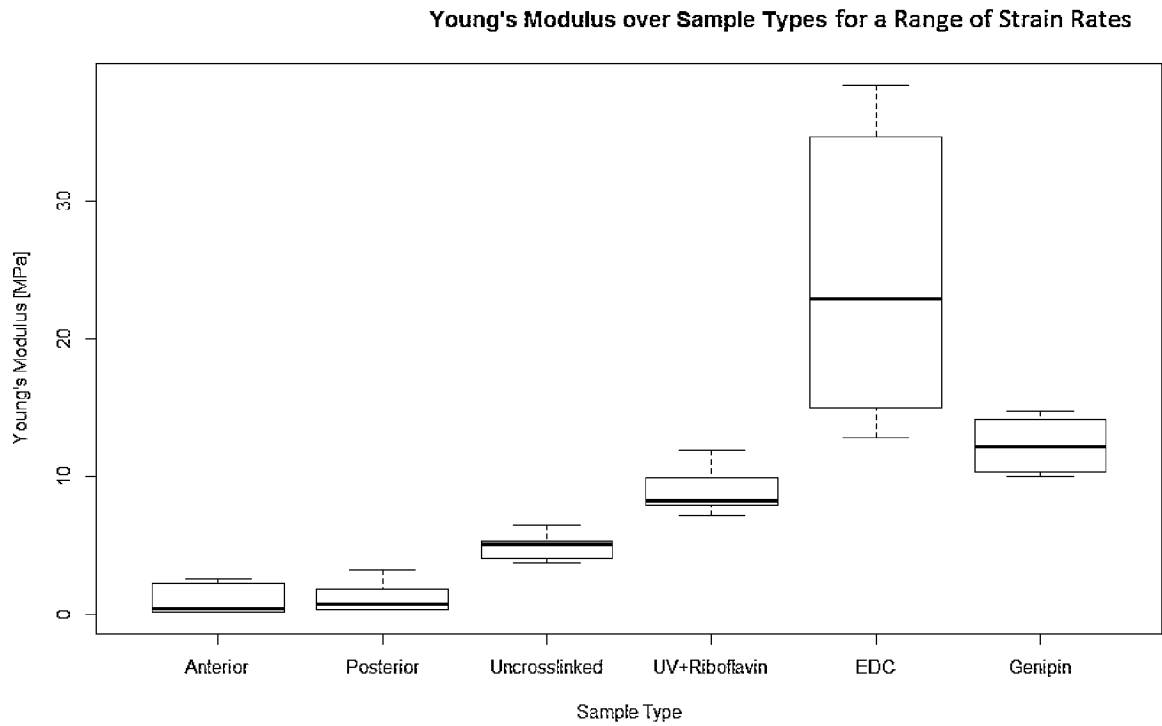


Figure 16

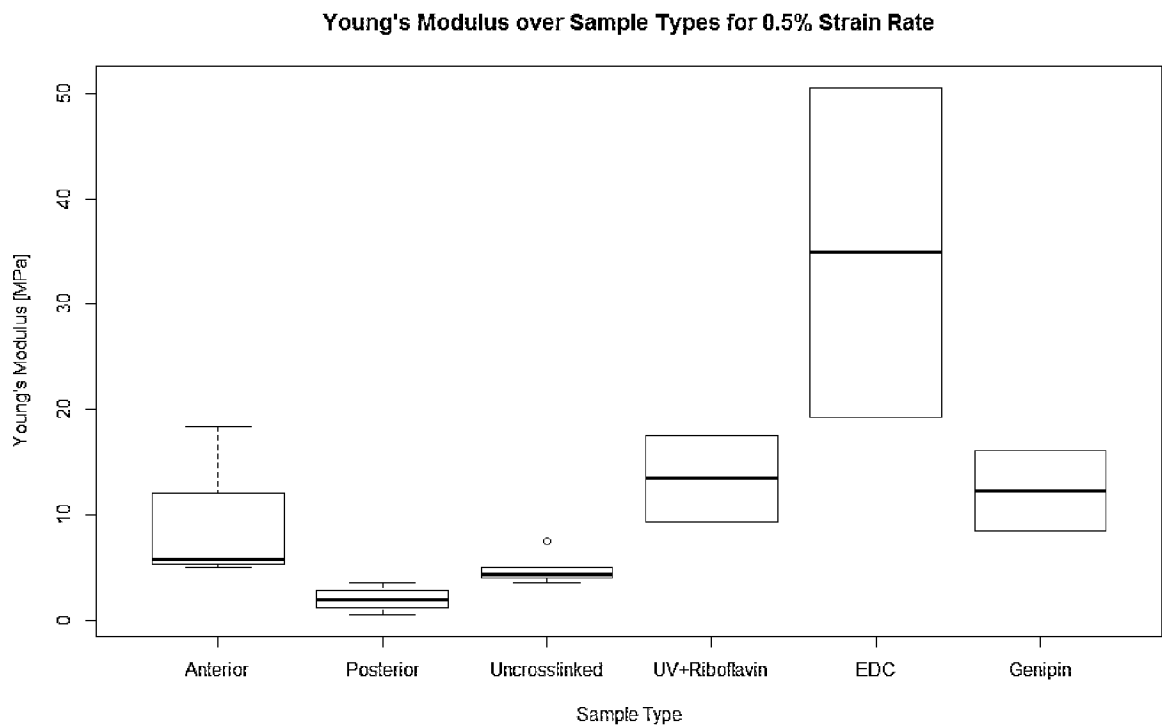


Figure 17

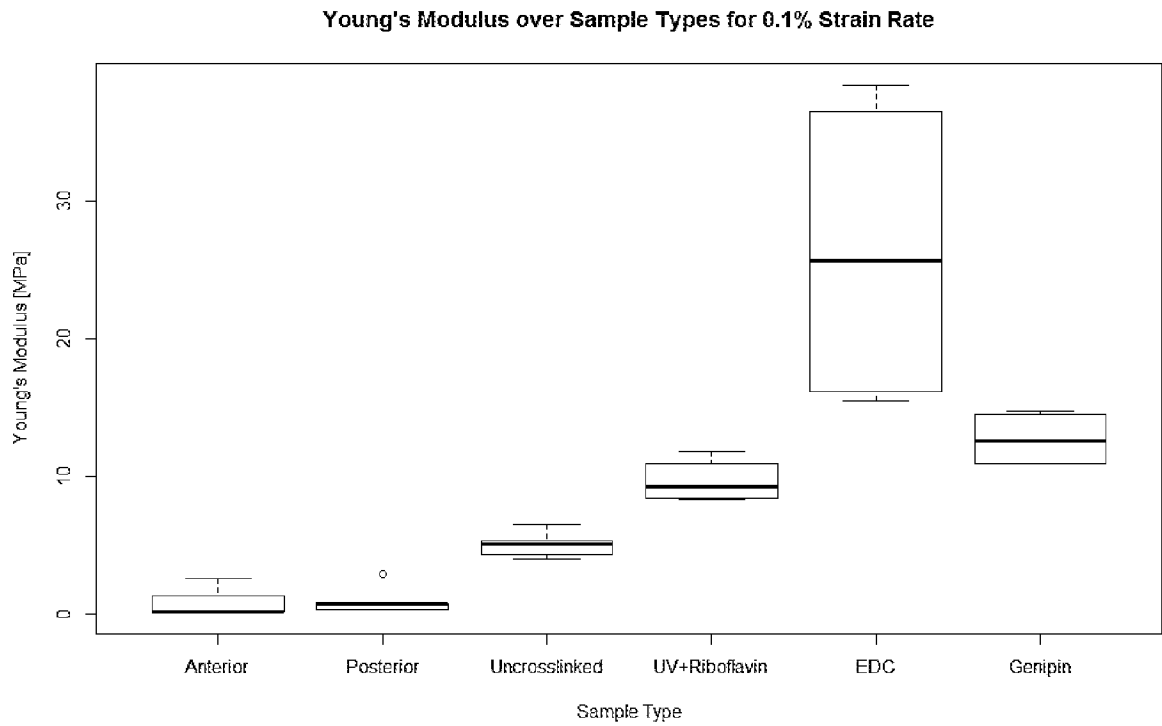


Figure 18

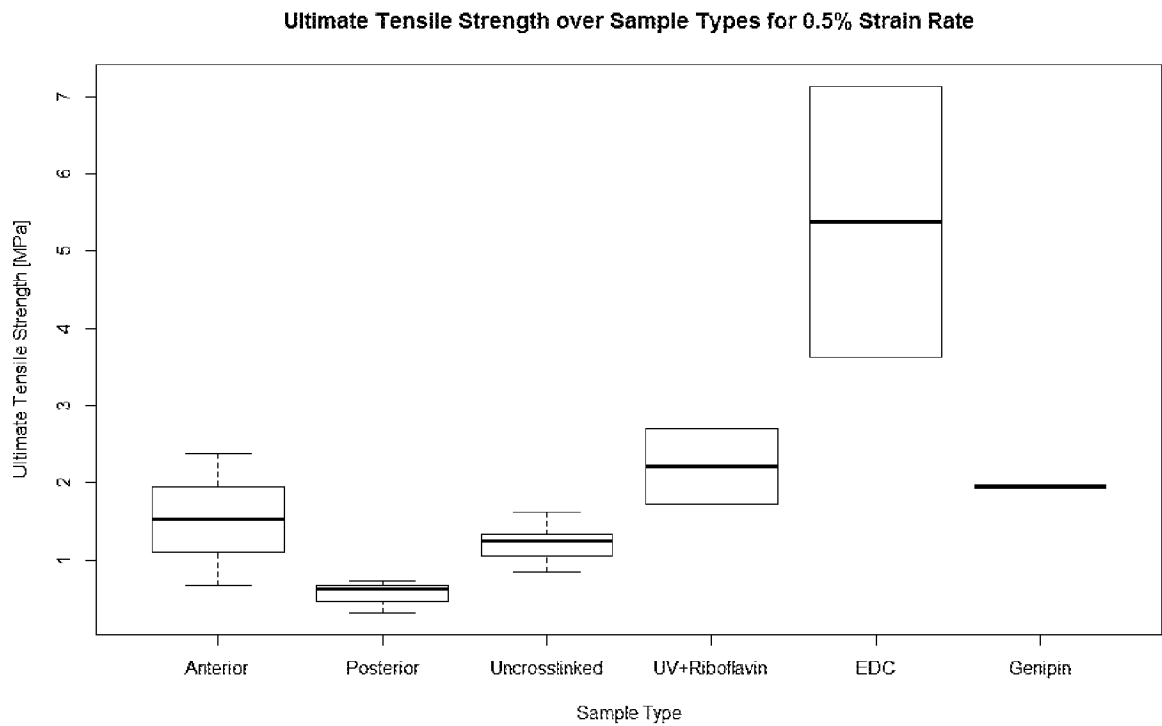


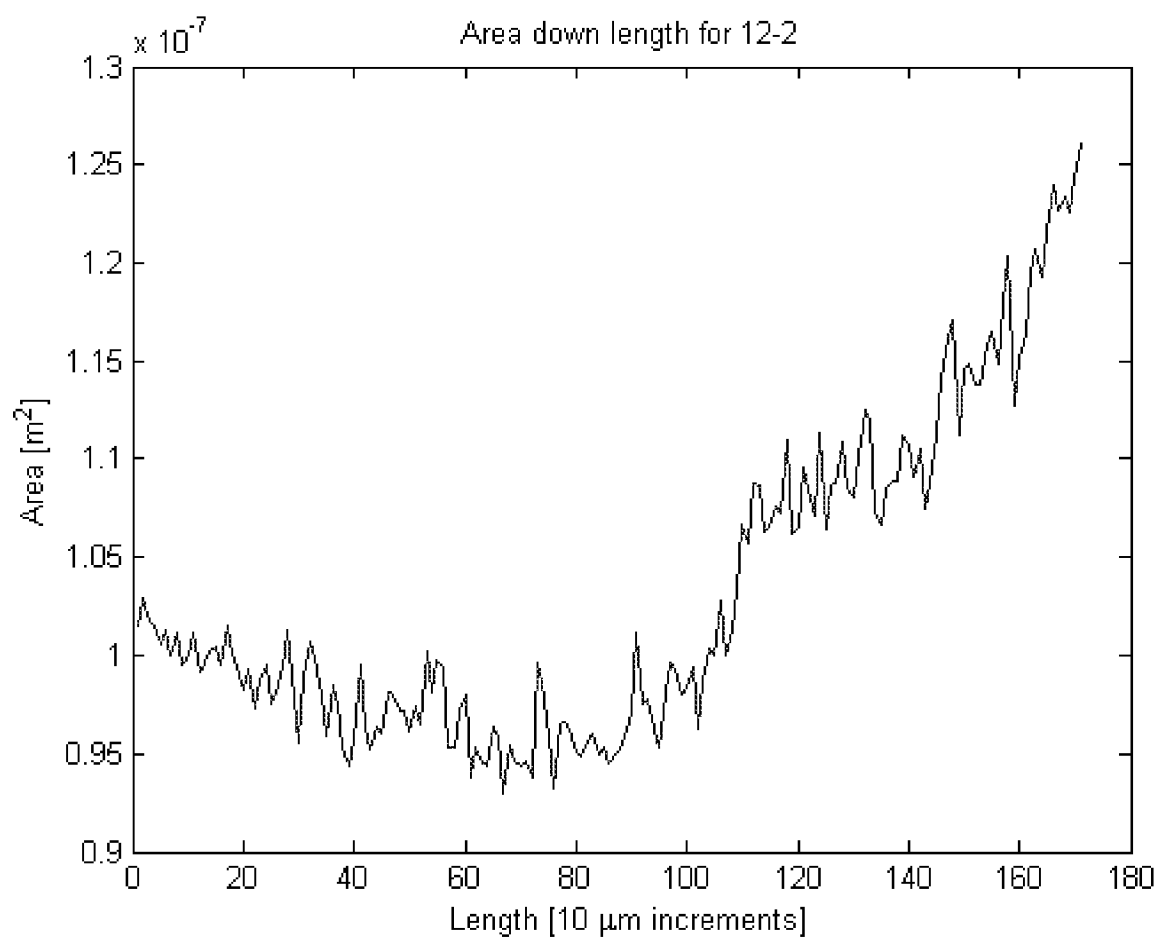
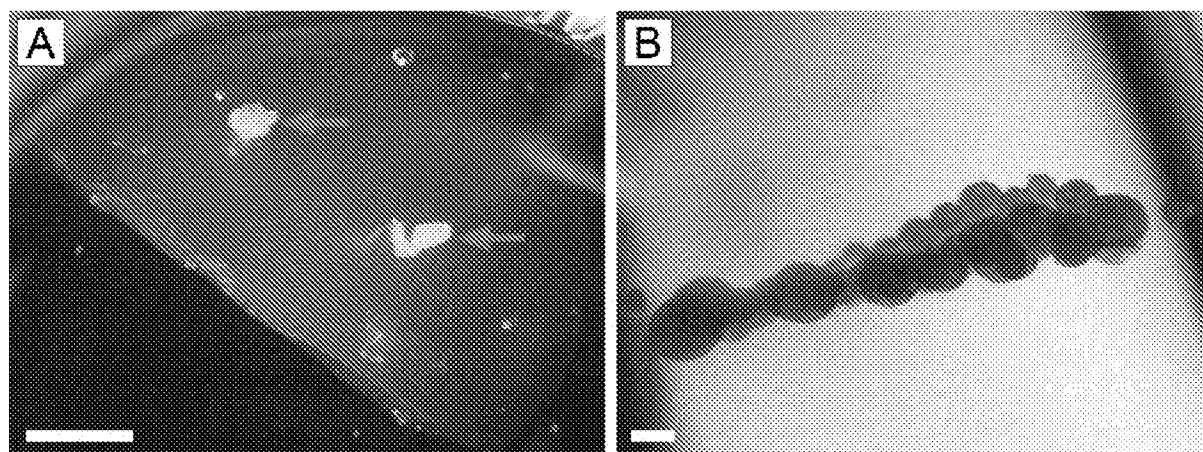
Figure 19**Figure 20**

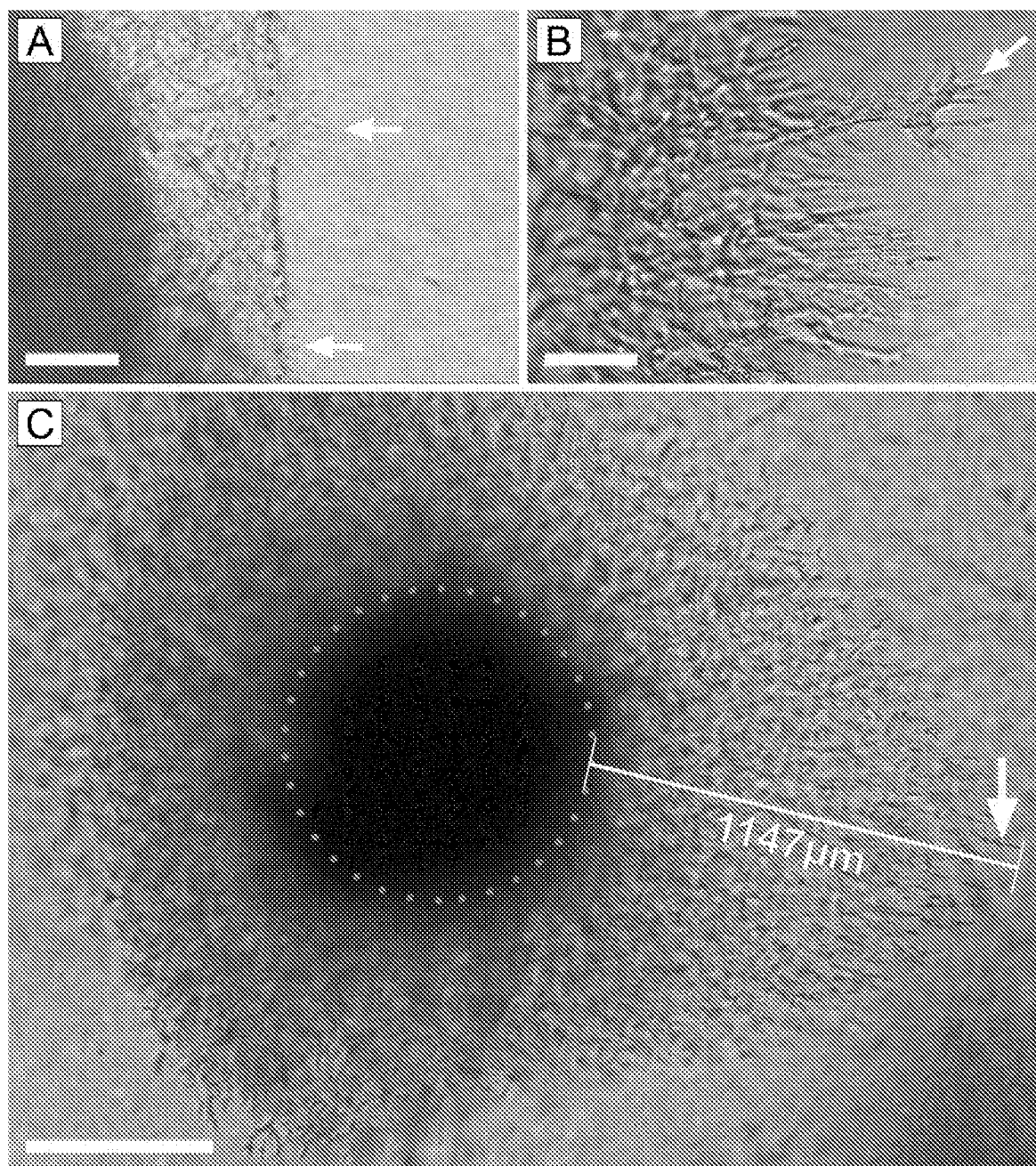
Figure 21

Figure 22

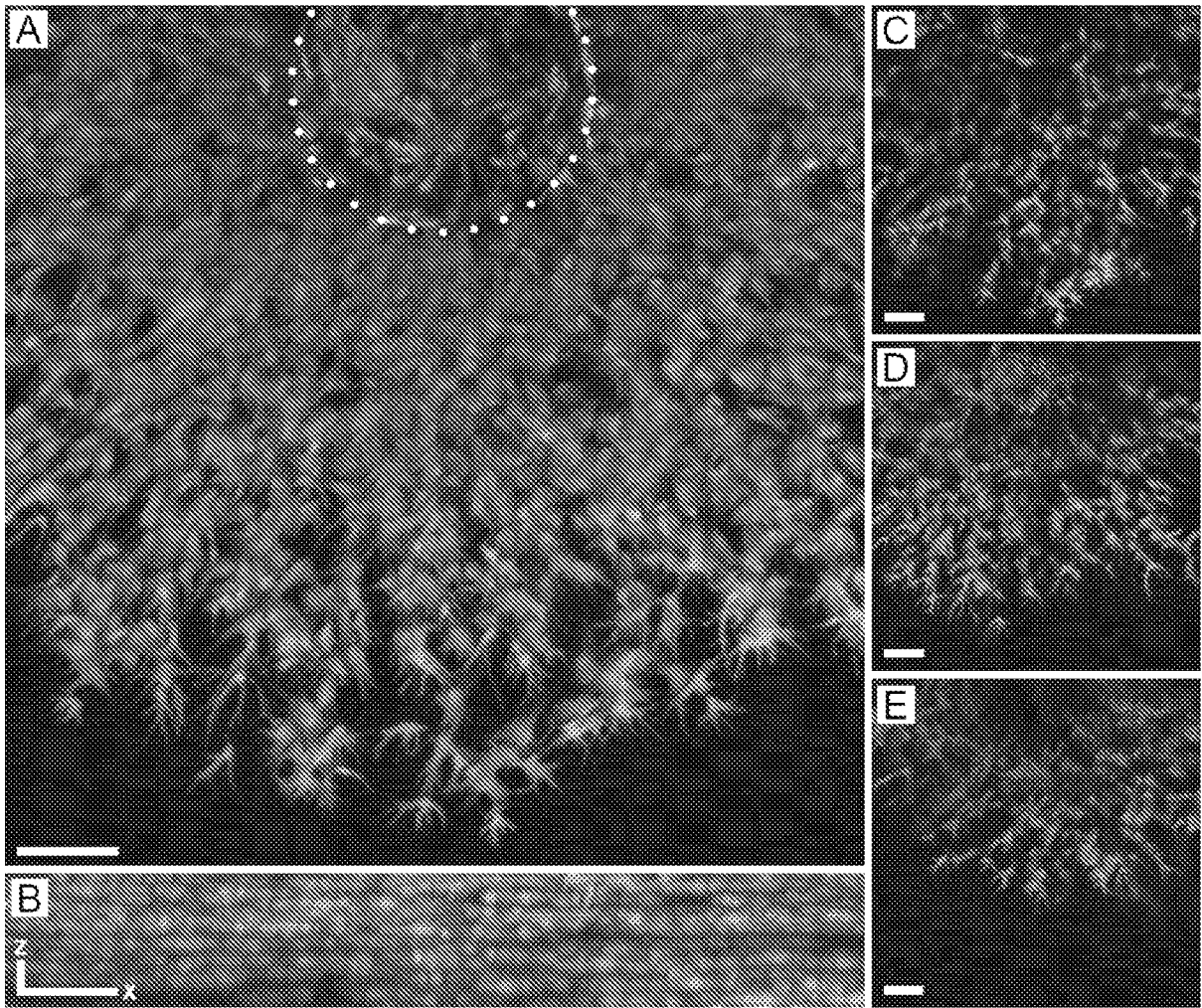


Figure 23

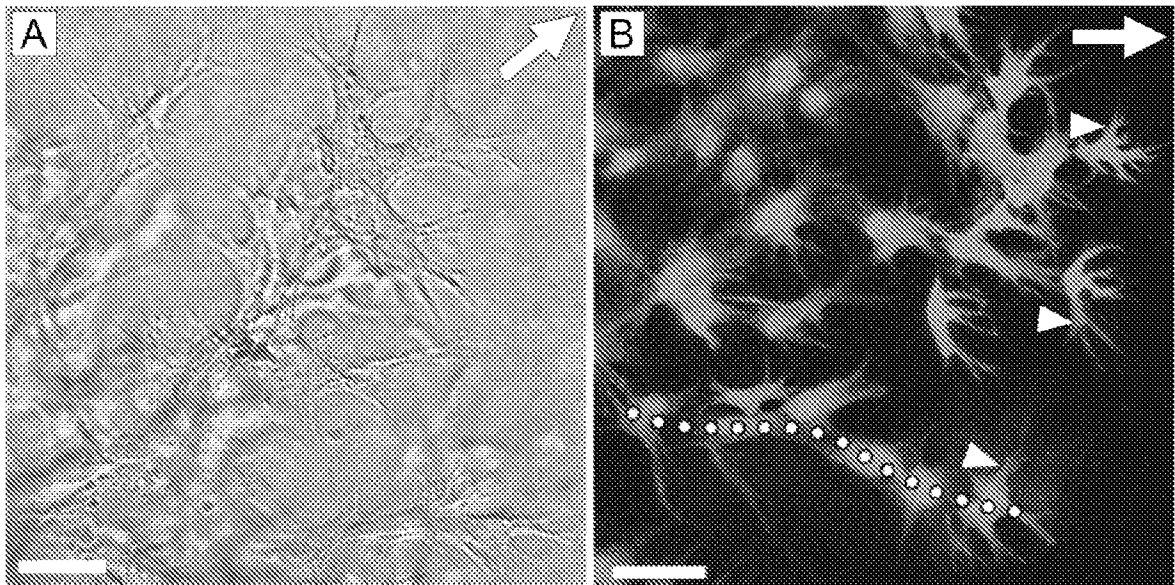


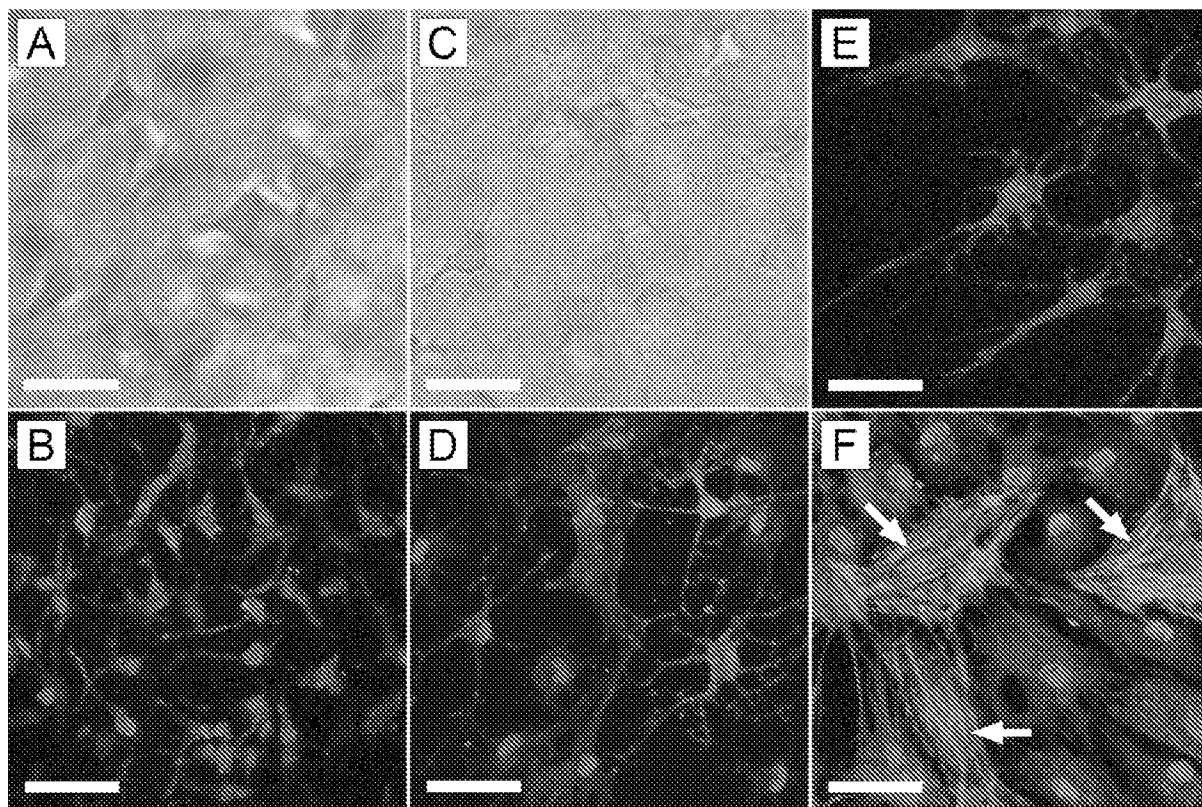
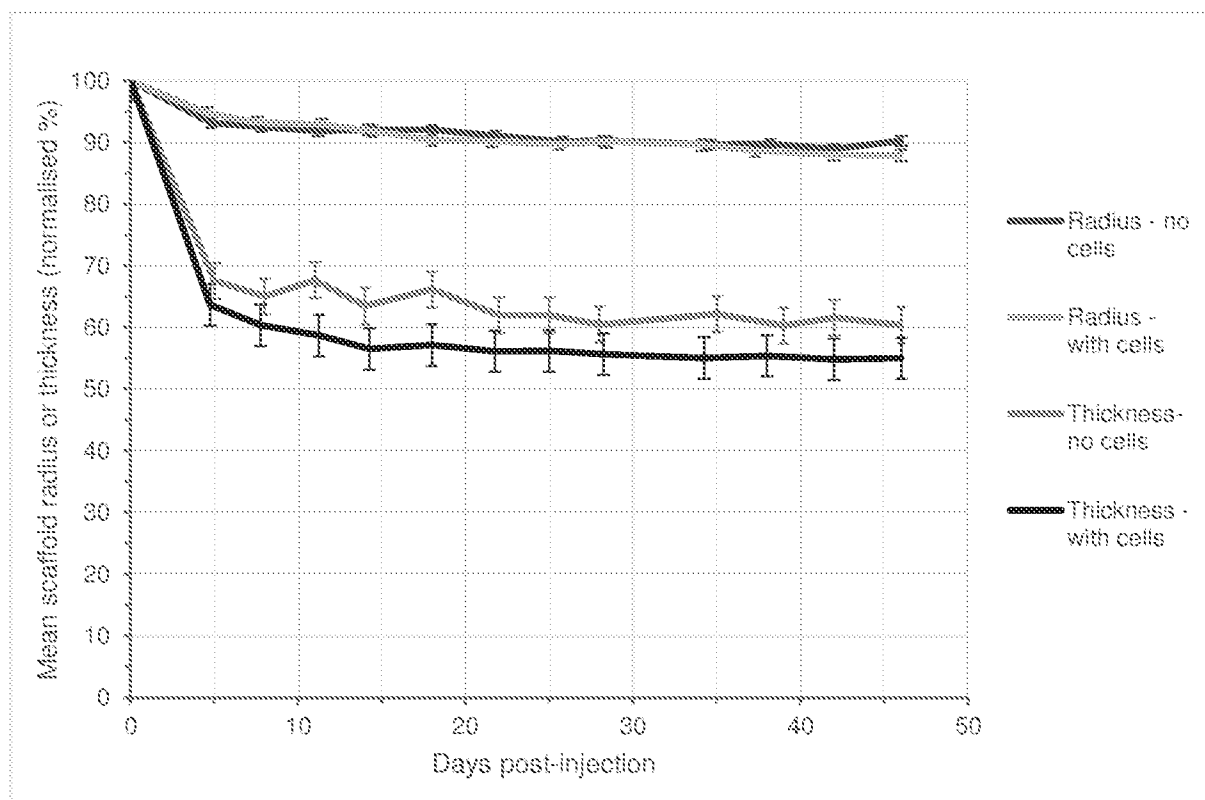
Figure 24**Figure 25**

Figure 26

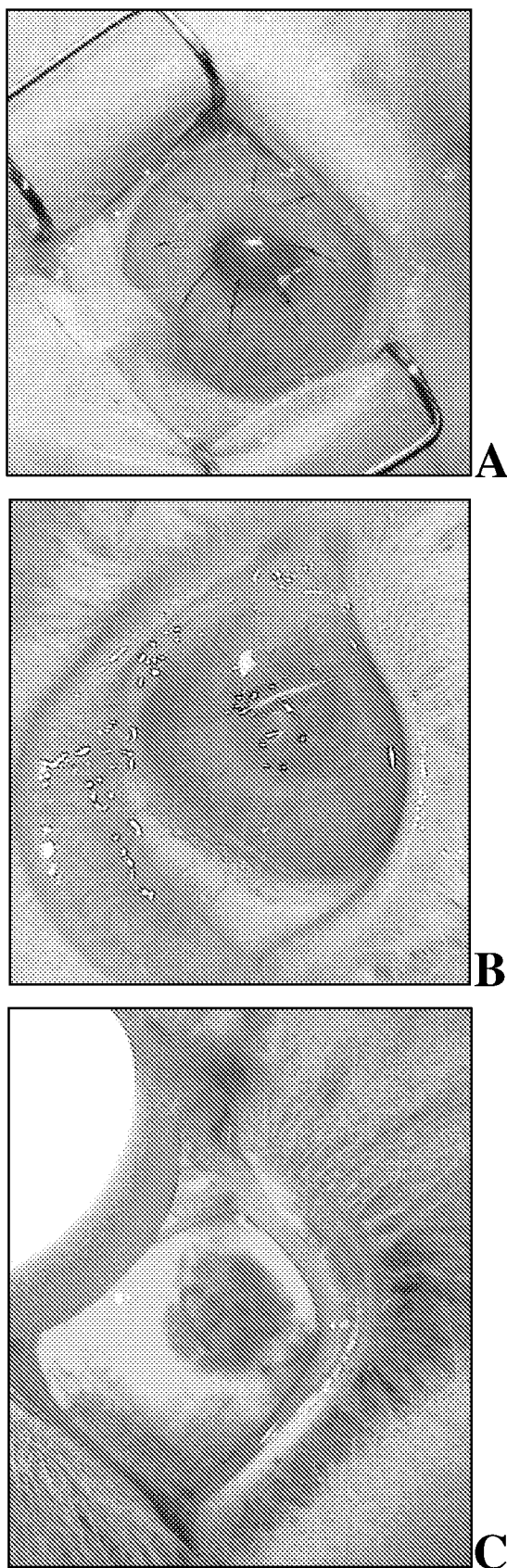


Figure 27

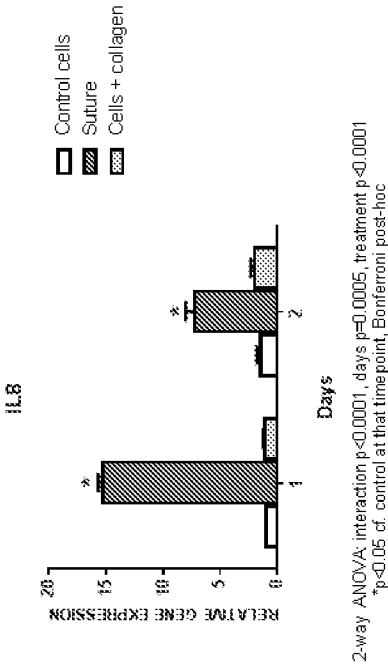
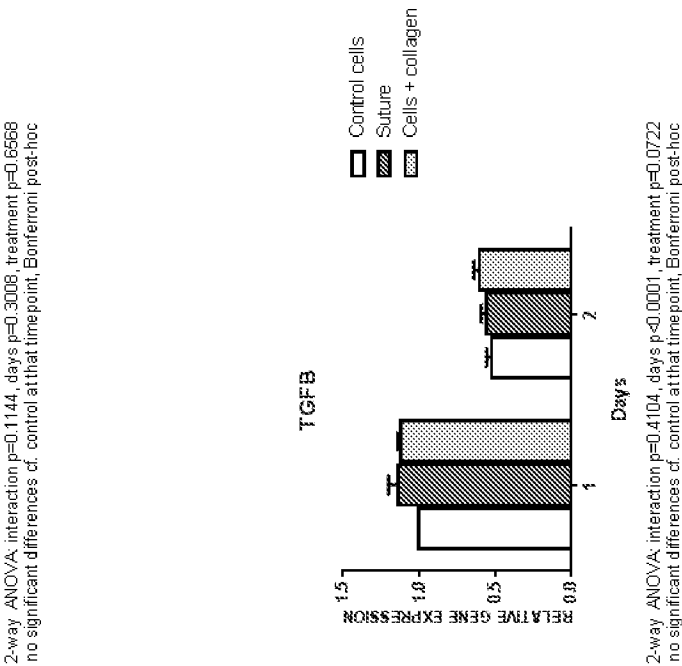
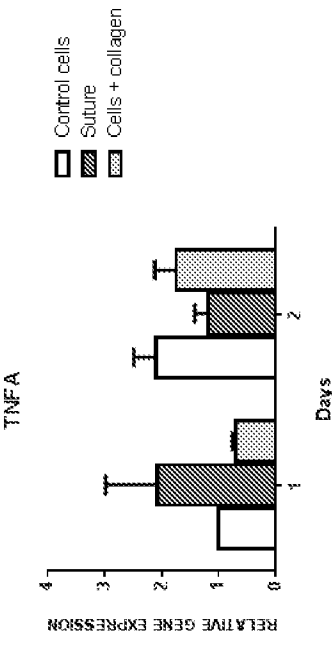


Figure 28

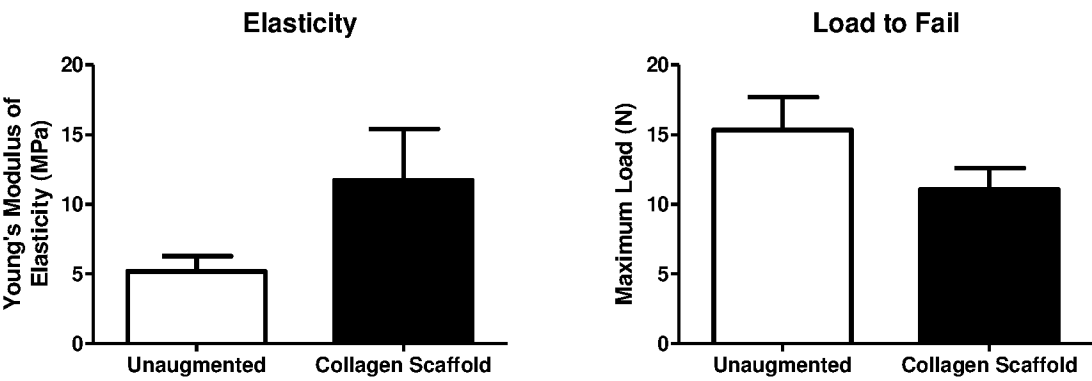


Figure 29

