(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 January 2019 (17.01.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/012295 A1

(51) International Patent Classification:
A61L 27/22 (2006.01) *A61L 27/36* (2006.01)

(21) International Application Number:
PCT/GB2018/052002

(22) International Filing Date:
13 July 2018 (13.07.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1711360.6 14 July 2017 (14.07.2017) GB

(71) Applicant: **RAFT ENTERPRISES LTD.** [GB/GB]; Leopold Muller Building, Mount Vernon Hospital, Northwood Middlesex HA6 2RN (GB).

(72) Inventors: **SAWADKAR, Prasad**; c/o Leopold Muller Building, Mount Vernon Hospital, Northwood Middlesex HA7 2RN (GB). **GARCÍA-GARETA, Elena**; c/o Leopold Muller Building, Mount Vernon Hospital, Northwood Middlesex HA6 2RN (GB).

(74) Agent: **CARRIDGE, Andrew**; Reddie & Grose LLP, The White Chapel Building, 10 Whitechapel High Street, London Greater London E1 8QS (GB).

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

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

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: TISSUE SCAFFOLD

(57) Abstract: There is provided a tissue scaffold and a method for making a tissue scaffold. The tissue scaffold comprises eiastin and optionally fibrin and/or collagen. The eiastin in the scaffold may be cross-linked. The eiastin that is cross-linked preferably comprises solubilised eiastin and is unfractionated.

WO 2019/012295 A1

TISSUE SCAFFOLD

This invention concerns tissue scaffolds, such as elastin-based tissue scaffolds and methods for forming such scaffolds.

Elastin is an extracellular structural protein found in connective tissues such as skin, adipose, lung, tendon, ligament, arteries, or cartilage. Its primary function is to retain the shape of tissues after stretching or contraction and has load bearing properties (Banga, 1966; Gray, 1973). *In vivo*, elastin forms by the process of elastogenesis, through the assembly and cross-linking of the protein tropoelastin (encoded by the ELN gene).

Tropoelastin typically consists of hydrophobic domains with many Gly, Val, Ala and Pro residues which often occur in repeats of several amino acids, such as Gly-Val-Gly-Val-Pro, Gly-Val-Pro-Gly-Val and Gly-Val-Gly-Val-Ala-Pro; and hydrophobic domains with many Lys and Ala residues which are important in cross-linking. Cross-linking of tropoelastin to form elastin is facilitated by lysyl oxidase.

Elastin is one of the most stable and abiding proteins in humans with a half-life of 74 years. Its excellent structural and biological properties has attracted attention for tissue engineering applications (Daamen *et al.*, 2007). For example, elastin provides elasticity to tissues and organs, and is abundant where elasticity is of primary importance, such as blood vessels, ligaments, in lung and in skin. However, elastin is a highly insoluble protein therefore, it remains a challenge to use it as a biomaterial (Leach *et al.*, 2005).

To overcome this challenge many existing strategies have been developed for α-elastin, a form of soluble elastin obtained following hydrolysis with oxalic acid. However, this process is expensive, time consuming and the total yield is minimal. Consequently, its clinical translation to a scaffold is questionable.

Some studies have reported mixing insoluble elastin with other materials, such as collagen (Ryan and O'Brien, 2015). However, the resulting scaffolds have weaker mechanical properties and altered biological responses compared to collagen itself.

The inventors report novel and economical, biologically active elastin-based materials and methods for their fabrication.

This invention concerns the formation of a scaffold by cross-linking a composition comprising elastin, such as solubilised elastin.

The invention also concerns a tissue scaffold comprising cross-linked elastin.

According to the invention, there is provided a method for forming a tissue scaffold comprising cross-linking a composition comprising solubilised elastin.

According to the invention, there is provided a method comprising cross-linking a composition, wherein the composition comprises elastin that has been contacted by a solubilising agent that can solubilise the elastin.

According to the invention, there is provided a method comprising: a) contacting elastin with a solubilising agent that is able to solubilise the elastin; and b) cross-linking the elastin composition formed in step a).

According to the invention, there is provided a tissue scaffold comprising cross-linked, solubilised elastin.

The elastin may be extracted or derived from a natural source. For example, the elastin may be derived from a mammalian source. The mammalian source may be a bovine source, such as bovine neck ligament, or a human source. Alternatively, the elastin may be recombinant elastin.

Elastin is a highly insoluble protein due to inter-chain cross-links. However, it can be solubilised (Daamen (2007)). Solubilised elastin is also referred to as hydrolysed elastin or elastin peptides.

Common methods of solubilising elastin include treating it with 0.25 M oxalic acid at 100°C, or treating it with 1M KOH in 80% ethanol. In addition, proteolytic enzymes capable of degrading elastic fibres, including serine-type elastases from polymorphonuclear leukocytes and several metallo-elastases of monocyte/macrophage origin, also result in solubilised elastin. Examples of hydrolysed forms of elastin are show in the table below.

Type	Preparation Method	Molecular mass
α	Oxalic acid solubilisation	Heterogeneous mixture, average 60 kDa
β	Oxalic acid solubilisation	Heterogeneous mixture, average 3-10 kDa
κ	KOH solubilisation	Heterogeneous mixture, average 70 kDa
PSP	Pepsin solubilisation	Heterogeneous mixture, average 25 KDa
ASP	Acid solubilisation	Heterogeneous mixture, average 25 kDa.
ESP	Elastase solubilisation	Heterogeneous mixture

Elastin peptides obtained after oxalic acid hydrolysis can be coacervated after suspension in 10 mM sodium acetate with 10 mM NaCl set to pH 5.5 with acetic acid, followed by heating and centrifugation at 37°C. As a result of this, two fractions are formed, α-elastin (a viscous coacervate) and β-elastin (in the supernatant).

The prior art has focussed mainly on using insoluble elastin in combination with other components, such as collagen, or has focussed on the α-elastin soluble component obtained following hydrolysis with oxalic acid and separation from β-elastin. Surprisingly, however, the inventors have found that solubilising elastin, and cross-linking the product of that solubilisation step, can form a promising and cost-effective tissue scaffold. So, there is no requirement to separate or isolate fractions of elastin, such as separating or isolating α-elastin and β-elastin. The solubilised elastin that is cross-linked may thus be considered crude or unfractionated. Advantageously, the invention may avoid the time, inconvenience and expense associated with isolating the α-elastin fraction. The invention may also improve total yield, as a step to separate α-elastin and β-elastin can be avoided.

According to the invention, there is provided a method for forming a tissue scaffold comprising cross-linking a composition comprising unfractionated solubilised elastin.

According to the invention, there is provided a method comprising cross-linking a composition comprising elastin, wherein the elastin is unfractionated and the elastin comprises solubilised elastin. The method may involve contacting elastin with a solubilising agent that is able to solubilise at least some of the elastin and then cross-linking the resulting composition.

The unfractionated elastin may thus be crude elastin in which there has been no purification, isolation, separation or refinement of one or more elastin fractions, or one or more different forms of elastin which result from the contact of elastin with a solubilising

agent. For instance, there may not have been isolation of one or more soluble elastin fractions, or separation of an insoluble elastin fraction from a soluble elastin fraction. Consequently, the composition may comprise different soluble forms of elastin. The composition may comprise elastin that has not been solubilised. The composition may thus comprise both soluble and insoluble forms of elastin. For example, following contact with the solubilising agent, the elastin may not be subjected to centrifugation. There may be no fractionation, purification, isolation, separation or refinement of the elastin following contact with the solubilising agent.

Surprisingly, the inventors have appreciated that an effective elastin-based tissue scaffold can be formed without requiring fractionation of elastin, in which one or more fractions of elastin are isolated and the isolated fraction(s) are subsequently used to form a scaffold. For example, the invention may not require isolation and utilisation of an α-elastin fraction. Advantageously, the present invention may not require conventional steps to fractionate the elastin, such as centrifugation and/or coacervation. Also advantageously, compositions comprising both soluble and insoluble elastin may be used. This may provide a significant benefit over known methods. For example, US2004/0136977 requires isolation of water-soluble elastin, involving centrifugation. JP2014183886 requires sequential rounds of acid fractionation of insoluble elastin, involving centrifugation.

The invention does not encompass methods comprising cross-linking of tropoelastin by, for example, the cross-linking of tropoelastin by lysyl oxidase, or the products of such methods.

Methods of the invention may comprise the step of solubilising the elastin. This may involve contacting the elastin with a solubilising agent that is able to solubilise at least some of the elastin. So, the invention may provide a method comprising: a) solubilising elastin to form a composition comprising unfractionated, solubilised elastin; b) cross-linking the product obtained from step a).

According to the invention, there is provided a method comprising: a) solubilising elastin to form a composition comprising solubilised elastin; b) cross-linking the composition obtained from step a).

According to the invention, there is provided a method comprising: a) contacting elastin with a solubilising agent to form a composition comprising solubilised elastin; and b) cross-linking the composition obtained from step a).

According to the invention, there is provided a method comprising cross-linking elastin that has been contacted with a solubilising agent, wherein the elastin has not been fractionated.

According to the invention, there is provided a method comprising cross-linking a composition, the composition comprising elastin, wherein the elastin is unfractionated and comprises solubilised elastin.

According to the invention, there is provided a tissue scaffold comprising cross-linked unfractionated solubilised elastin.

According to the invention, there is provided a tissue scaffold comprising cross-linked elastin, wherein the composition comprising cross-liked elastin has been formed by cross-linking a formulation comprising elastin that comprises solubilised elastin and wherein the elastin has not been fractionated.

According to the invention, there is provided a tissue scaffold comprising cross-linked elastin, wherein the elastin has been contacted with a solubilising agent and has not been fractionated.

The scaffold may be prepared from a solution comprising 1 to 20% (w/v) elastin, for example 5 to 15% (w/v) elastin, such as around 10% (w/v) elastin.

Preferably, the elastin is, or has been, solubilised by contacting with acid, most preferably oxalic acid.

According to the invention, there is provided a method of solubilising elastin comprising contacting elastin with a solubilising agent that is able to solubilise at least some of the elastin. The solubilising agent is preferably an acid, more preferably oxalic acid.

According to the invention, there is provided a method of solubilising elastin comprising contacting elastin with an acid, preferably oxalic acid.

In a particularly preferred embodiment, the elastin is solubilised at a temperature less than 100°C, preferably at a temperature of less than or equal to 50°C, more preferably at a temperature of 15 to 30°C, such as room temperature.

The acid, preferably oxalic acid, may be at less than 1M, preferably less than 0.75M, more preferably at 0.5M, or less than 0.5M. The acid may be at least 0.25M. For example, the acid may be at 0.2M to 1M, for example 0.25M to 0.75M.

The method of solubilising elastin, as described herein, is contrary to the established method of solubilising elastin using oxalic acid. The conventional method of solubilising elastin using oxalic acid is carried out at 100°C (see, for example, Daamen et al. (2007)). However, the inventors have found that effective solubilisation, for the purposes of forming a scaffold of the invention, may occur at temperatures less than 100°C. Although not wishing to be bound by theory, the inventors have postulated that the treatment with oxalic acid at temperatures less than 100°C may lead to formation of a mixture comprising α- and β-elastin. If this is the case, methods of the invention may comprise cross-linking α- and β-elastin, and scaffolds of the invention may comprise cross-linked α- and β-elastin. Advantageously, the inventors have appreciated that it is not necessary to separate solubilised fractions, such as isolating the α-elastin fraction, and that a crude, or unfractionated, mixture of solubilised elastin can be used to form an effective scaffold. It is appreciated that compositions comprising solubilised elastin, such as unfractionated solubilised elastin, may comprise some elastin that has not been solubilised. The composition may thus comprise a mixture of insoluble elastin and soluble elastin.

The invention may thus provide a method comprising cross-linking a composition comprising soluble elastin and insoluble elastin. The invention may thus provide a tissue scaffold comprising cross-linked elastin, wherein the elastin comprises soluble elastin and insoluble elastin.

Surprisingly, the inventors have appreciated that complete solubilisation of the elastin to be cross-linked, may not be required to obtain an effective tissue scaffold.

According to the invention, there is provided a method comprising cross-linking a composition comprising insoluble elastin.

According to the invention, there is provided a tissue scaffold comprising cross-linked, insoluble elastin.

Solubilisation, or contact with acid, preferably takes place for at least 30 seconds, more preferably at least one minute. For example, the solubilisation or contact with acid may take place for about 1 to 3 minutes. The solubilisation, or contact with acid, may take place for up to 5 minutes. This contrasts with the conventional treatment of elastin with oxalic acid which typically takes place for about 1 hour (see, for instance, US2004/0136777).

According to the invention there is provided a method comprising contacting elastin with acid, preferably oxalic acid. Preferably, contact with the acid takes place at a temperature less than 100°C, most preferably at a temperature of less than or equal to 50°C, more preferably at a temperature of 15 to 30°C, such as room temperature or ambient temperature. The method may further comprise cross-linking the resulting product.

According to the invention, there may be provided a method of forming a tissue scaffold comprising cross-linking a composition comprising α-elastin and β-elastin.

According to the invention, there may be provided a tissue scaffold comprising a cross-linked composition comprising α-elastin and β-elastin.

Cross-linking may occur using any one of a number of cross-linking agents or cross-linking techniques commonly known to those skilled in the art, such as chemical, radiation and dehydrothermal methods.

References herein to "cross-linking" concern covalent cross-linking. Preferably, cross-linking is achieved non-enzymatically, using a chemical cross-linking agent.

Cross-linking may occur in the presence of the solubilising agent (e.g. acid such as oxalic acid). So, the invention may provide, or make use of, a composition comprising elastin, a solubilising agent and a cross-linking agent.

Examples of suitable chemical cross-linking agents include: carbodiimide coupling agents such as N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) ; N- hydroxysuccinimide (NHS) , azide coupling agents; diisocyanate cross-linking agents such as hexam ethylene diisocyanate; epoxide cross-linking agents such as epi- chlorhydrin, glycidylethers and

glycidylamines; and aldehyde cross-linking agents such as formaldehyde, glutaraldehyde and glyoxal.

The chemical cross linking agent may comprises N- (3- dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and/or N-hydroxysuccinimide (NHS) .

The chemical cross linking agent may comprise aldehyde cross-linking agents such as formaldehyde, glutaraldehyde and glyoxal. Aldehyde cross-linking agents may have the advantage of providing extracellular matrix compositions with improved biocompatibility. In a preferred embodiment, the aldehyde cross-linking agent is glutaraldehyde. The use of glutaraldehyde as a cross-linking agent may provide an advantage of yielding an optimal cross-link density more rapidly than other aldehydes and is also capable of achieving a relatively high density of cross-linking. In a preferred example, the chemical cross-linking agent is glutaraldehyde.

During the cross-linking step, the cross-linking agent may be present in an amount of about 0.2 to 5% (v/v), such as 0.5 to 3% (v/v), preferably 0.5 to 1.5% (v/v), e.g. 1% (v/v).

When the cross-linking agent comprises glutaraldehyde or N- (3- dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and/or N-hydroxysuccinimide (NHS) , the method according to the invention may additionally comprise the addition of a toxicity reducing agent (e.g. lysine or sodium borohydride).

The step of cross-linking the composition comprising solubilised elastin may be carried out at a temperature of 20°C to 50°C, preferably about 37°C. Contact or incubation with the cross-linking agent may typically be performed between 1 minute and 24 hours (e.g. 4 hours). For example the cross-linking may take place for about an hour, or at least one hour. The cross-linking may take place in the presence of CO_2, for example at least 2% CO_2 (by volume), for example 2 to 10% CO_2 (by volume), or at about 5% CO_2 (by volume).

According to the invention, there is provided a composition comprising elastin, a solubilising agent for solubilising elastin, and a cross-linking agent.

Methods of the invention may comprise casting the composition comprising solubilised elastin. Casting may comprise applying the composition comprising solubilised elastin to a mould of a predetermined shape. The casting may occur prior to or during cross-linking.

It is preferred that methods of the invention comprise lyophilisation following cross-linking. For example, the composition may be frozen at -80°C, preferably overnight, and then lyophilised for about 48 hours. Preferably, lyophilisation occurs for at least 24 hours.

According to the invention, there is provided a method comprising lyophilising a composition comprising cross-linked elastin. According to the invention, there is provided a method comprising lyophilising a composition comprising solubilised and cross-linked elastin. For example, there is provided a method comprising lyophilising a composition, the composition comprising cross-linked unfractionated solubilised elastin.

According to the invention, there is provided a method of forming a tissue scaffold comprising lyophilising a composition comprising cross-linked elastin, wherein the composition comprising cross-liked elastin has been formed by cross-linking a formulation comprising elastin that is unfractionated and that comprises solubilised elastin.

According to the invention, there is provided a method comprising:
a) solubilising elastin; b) cross-linking solubilised elastin obtained from step a); and c) lyophilising the product from step b).

According to the invention, there is provided a method comprising:
a) solubilising elastin; b) cross-linking unfractionated solubilised elastin obtained from step a); and c) lyophilising the product from step b).

According to the invention, there is provided a method comprising: a) contacting elastin with a solubilising agent that is able to solubilise the elastin to form a composition comprising solubilised elastin; b) cross-linking the composition produced in step a); and c) lyophilising the product of step b).

According to the invention, there is provided a tissue scaffold comprising lyophilised, cross-linked elastin.

According to the invention, there is provided a tissue scaffold comprising lyophilised, cross-linked, solubilised elastin.

According to the invention, there is provided a tissue scaffold comprising lyophilised, cross-linked unfractionated solubilised elastin.

According to the invention, there may be provided a method comprising lyophilising a composition comprising cross-linked α-elastin and β-elastin.

According to the invention, there may be provided a tissue scaffold comprising lyophilised, cross-linked α-elastin and β-elastin.

According to the invention there may be provided a tissue scaffold comprising lyophilised, cross-linked elastin, wherein the cross-linked elastin has been formed by cross-linking a composition comprising soluble elastin and insoluble elastin.

Methods of the invention may comprise washing or cleaning to remove agents involved in solubilising and/or cross-linking. Washing preferably takes place following solubilisation. This may include contacting or washing with a reducing agent, particularly if the cross-linking agent comprises an aldehyde cross-linking agent. Washing may comprise ultrasonic cleaning. For example, the scaffold may be washed using water in an ultrasonic cleaner.

The presence of the reducing agent may stabilise the cross-linking process and result in a scaffold with enhanced biological efficacy. Furthermore, the presence of the reducing agent is likely to reduce the cytotoxic effects caused by the leaching of un-reduced cross-linking agent from the composition.

Examples of a suitable reducing agent include sodium borohydride or agents with similar carbonyl group reactivity. The reducing agent may typically be added in an amount of 0.1% w/v to 10% w/v (e.g. about 1% w/v) .

The step of washing to remove agents involved in solubilising and/or cross-linking may be carried out for at least 5 hours, preferably at least 8 hours. For example, to remove oxalic acid and unbound glutaraldehyde, the scaffold may be washed with a reducing agent (such as sodium borohydride) for approximately 8 hours. Preferably, the scaffold is agitated or shaken whilst in contact with the reducing agent.

After contact with the reducing agent, there may be a further washing step, which may involve washing with water e.g. distilled water, and/or ethanol. This may help to remove any remaining unbound cross-linking agent or oxalic acid.

According to the invention, there is provided a method comprising:
a) solubilising elastin; b) cross-linking a composition comprising solubilised elastin obtained from step a); c) lyophilising a product from step b); and d) washing the product from step c).

According to the invention there is provided a method comprising:
a) solubilising elastin; b) cross-linking a composition comprising unfractionated, solubilised elastin obtained from step a); c) lyophilising the product from step b); and d) washing the product from step c).

According to the invention, there is provided a method comprising: a) contacting elastin with a solubilising agent that is able to solubilise the elastin; b) cross-linking the composition obtained from step a); c) lyophilising the product obtained from step b); and d) washing the product of step c).

After washing, the scaffold may be sterilised. In some embodiments, sterilisation involves washing the scaffold with ethanol and PBS.

According to the invention there is provided a method comprising: a) solubilising elastin; b) cross-linking the composition obtained from step a); c) lyophilising the product from step b); d) washing the product from step c); and e) sterilising the product from step d).

According to the invention there is provided a method comprising: a) solubilising elastin; b) cross-linking a composition comprising unfractionated, solubilised elastin obtained from step a); c) lyophilising the product from step b); d) washing the product from step c); and e) sterilising the product from step d).

According to the invention, there is provided a method comprising: a) contacting elastin with a solubilising agent; b) cross-linking the composition produced by a); c) lyophilising the product of step b); d) washing the product of step c); and e) sterilising the product of step d) Scaffolds of the invention are preferably sterile.

According to the invention, there is provided a tissue scaffold obtained or obtainable by a method according to the invention.

In some embodiments, scaffolds of the invention are not hydrogels.

It is envisaged that the methods and scaffolds of the invention may also be applicable to elastin derivatives or fragments, such as synthetic elastin sequence-based materials or elastin-like peptides (ELPs). ELPs are biopolymers based on key, repeating elastin sequences. For example, ELPs may have repeating peptides, such as pentapeptides or hexapeptides comprising Val, Gly and/or Pro. ELPs may possess the elastic properties of elastin using the pentapeptide repeat VPGXG where X is any amino acid besides proline (such as Val or Ile) (Zhang et al (2015), Daamen (2007)).

According to the invention, there may be provided a method comprising cross-linking elastin derivatives or fragments.

According to the invention, there may be provided a tissue scaffold comprising cross-linked elastin derivatives or fragments.

According to the invention, there may be provided a method comprising lyophilising cross-linked elastin derivatives or fragments.

According to the invention there may be provided a tissue scaffold comprising lyophilised cross-linked elastin derivatives or fragments.

Scaffolds of the invention may comprise other extracellular matrix components.

Scaffolds of the invention may comprise collagen. Consequently, according to the invention, there is provided a scaffold comprising elastin and collagen.

Scaffolds of the invention may comprise fibrin. Consequently, according to the invention, there is provided a scaffold comprising elastin and fibrin.

Scaffolds of the invention may comprise collagen and elastin. Consequently, according to the invention, there is provided a scaffold comprising elastin, collagen and fibrin.

Preferably, the elastin has been solubilised. Preferably the elastin is unfractionated, solubilised elastin. The elastin may be unfractionated. The elastin may comprise solubilised elastin. The elastin may comprise insoluble elastin.

Scaffolds of the invention may be formed by mixing a composition comprising elastin with a) a composition comprising collagen; and/or b) a composition comprising fibrin.

The composition containing collagen may comprise a collagen hydrogel. For example, a collagen hydrogel may be formed by standard procedures. The collagen hydrogel may be prepared using 80% rat tail collagen type I and 10X Minimal Essential Medium, neutralised using 5M and 1M sodium hydroxide and added 10X DMEM (Dulbecco's Modified Eagle Medium).

The composition containing fibrin may contain a fibrin gel. The fibrin gel may be formed by standard procedures. The fibrin gel may be prepared with 2% fibrinogen dissolved in 1 ml of PBS, then adding 1% thrombin with 0.1M $CaCl_2$.

According to the invention, there is provided a method comprising mixing a composition comprising elastin (preferably a composition comprising unfractionated, solubilised elastin) with a composition comprising collagen (preferably a collagen hydrogel), and/or a composition comprising fibrin (preferably a fibrin gel).

According to the invention, there is provided a composition comprising elastin (preferably unfractionated, solubilised elastin), collagen and/or fibrin. The composition may comprise a cross-linking agent.

The composition comprising elastin is preferably mixed with the composition comprising collagen and/or the composition comprising fibrin, prior to a cross-linking step. Consequently, the resulting scaffold may comprise cross-linked elastin, cross-linked collagen and/or cross-linked fibrin.

According to the invention, there is provided a composition comprising elastin; a solubilising agent for solubilising elastin; a cross-linking agent; and fibrin and/or collagen.

The cross-linking may proceed as already described herein. For example, the cross-linking agent may comprise glutaraldehyde and may be carried out in the presence of CO_2. The cross-linking agent may be added to the composition comprising the elastin, collagen

and/or fibrinogen. Alternatively, the cross-linking agent may be added to the composition comprising elastin, the composition comprising collagen and/or the composition comprising fibrinogen, prior to mixing the compositions. For example, the composition comprising elastin may comprise the cross-linking agent. The concentration of the cross-linking agent in the composition comprising elastin may be at a level (e.g. 3% by volume) such that when the composition comprising elastin is mixed with the composition comprising collagen and/or the composition comprising fibrin, the concentration is at a desirable level for cross-linking to take place (e.g. about 1% v/v).

Prior to cross-linking, the composition may be cast, as described herein.

Once the cross-linking has taken place, the scaffold may be lyophilised and/or washed, as described herein.

The relative amounts of elastin, collagen and/or fibrinogen may be adjusted to impart different architectural, mechanical and biodegradation properties to the resulting scaffold. For example, scaffolds containing higher proportions of elastin may result in denser structural networks, greater elasticity and delayed degradation compared to scaffolds with lower proportions of elastin. Increasing the amount of fibrin may increase the mechanical strength and accelerate the biodegradation rate. Increasing the amount of collagen may also accelerate the biodegradation rate. Using particular combinations of elastin, collagen and/or fibrinogen may also allow enhancement of angiogenic properties of the scaffold.

According to the invention, there is provided a tissue scaffold according to the invention for use as a medicament.

According to the invention, there is provided a method of promoting tissue healing, regeneration or repair comprising applying a tissue scaffold according to the invention, to a patient. For example, the scaffold may be used in wound healing or tissue grafts (such as skin grafts). The scaffolds of the invention may be particularly applicable to soft tissue regeneration or repair, such as skin regeneration or vascular tissue regeneration. For instance, scaffolds may be used in adipose, skin, vascular grafts, heart valves or lung tissue engineering.

According to the invention, there is provided a tissue scaffold according to the invention, for use in promoting tissue healing, regeneration or repair.

According to the invention, there is provided use of a tissue scaffold according to the invention, in the manufacture of a medicament for promoting tissue healing, regeneration or repair

The invention may provide a method substantially as described herein with reference to the figures.

The invention may provide a tissue scaffold substantially as described herein with reference to the figures.

According to the invention, there is provided a scaffold as described herein, seeded with cells. The scaffold may be *in vitro* or *ex vivo*. The cells may be stem cells, such as human adipose-derived stem cells (hADSCs).

According to the invention, there is provided a method comprising seeding a scaffold of the invention with cells.

According to the invention, there may be provided a cell or tissue culture comprising a scaffold as defined herein.

Scaffolds of the invention may have a mean pore size less than 120 μm. Scaffolds of the invention may have a mean pore size less than 100 μm. Scaffolds of the invention may have a mean pore size of 10 μm or greater. Scaffolds of the invention may have a mean pore size of 20 μm or greater. For instance, scaffolds of the invention may have a mean pore size of 10 to 120 μm. Scaffolds of the invention may have a mean pore size of 20 to 100 μm.

The pore size distribution may be altered by including collagen and/or fibrin with elastin, or by changing the relative amounts of each component (see, for example, Figures 14 and 15).

Scaffolds of the invention may have a modal pore size of 80 μm or less. For example, the modal pore size may be 60 μm or less. The modal pore size may be 1 μm or greater, for example 10 μm or greater, or 20 μm or greater. For instance, the modal pore size may be in the range of 1 to 80 μm, for example 1 to 60 μm, 20 to 60 μm, or 1 to 60 μm.

In one example, the modal pore size may be in the range of 1-20 μm. In another example, the modal pore size may be in the range of 20 to 40 μm. In one embodiment, the modal pore size may be in the range 40 to 60 μm.

Characteristics of scaffolds such as pore size and porosity may be calculated using appropriate readily-available software. For example, ND ("Nearest Distance") is an ImageJ plugin that was developed to calculate the average size and distance between pores and their nearest neighbours in porous scaffolds (see Haeri et al. (2015)). DiameterJ is another example of an ImageJ plugin that can be used to measure pore parameters. Microscopic images of the scaffold (e.g. SEM images) may be used as input.

The total porosity of scaffolds of the invention may be at least 25%. For example, the total porosity may be at least 40%.

Examples of the invention are now described by way of example only, with reference to the accompanying drawings, in which:

Figure 1 shows elastin scaffold fabrication process from insoluble elastin (A), mixed with 0.5M oxalic acid (B), crosslinked with 1% GTA and incubated at 37°C for 1 hour (C), frozen at -80°C overnight (D), and lyophilised for 48 hours (E);

Figure 1 shows scaffolds fabricated without crosslinking agent (A), or with cross-linking agent (B);

Figure 2 shows a scaffold stabilisation study without crosslinking agent (A), and with crosslinking agent (B) after 28 days in PBS;

Figure 3 shows a live/dead assay at 1(A), 3 (B) and 7 (C) days for adipose derived stem cells (ADSC) growing on the scaffolds, with green points indicating alive cells;

Figure 4 shows a cell proliferation assay using alamar blue at 1, 3 and 7 days;

Figure 6 shows scanning electron microscopy (SEM) images of elastin scaffolds;

Figure 7 shows live/dead assay on days 1,3 and 7 for different combination scaffolds (3A= Collagen/ Elastin /Fibrin 2:1:1; 3B= Elastin/ Collagen/ Fibrin 2:1:1; 3C=Fibrin/ Collagen/ Elastin 2:1:1; 3D=Fibrin/ Collagen/ Elastin 1:1:1);

Figure 8 shows a cell proliferation assay (alamar blue activity) for combination scaffolds (3A= Collagen/ Elastin/ Fibrin 2:1:1; 3B= Elastin/ Collagen/ Fibrin 2:1:1; 3C=Fibrin/ Collagen/ Elastin 2:1:1; 3D=Fibrin/ Collagen/ Elastin; 1:1:1);

Figure 9 shows SEM Images illustrating differences in fibril network and pore structure of each individual combination, A) 3A= Collagen/ Elastin/ Fibrin 2:1:1, B) 3B= Elastin/ Collagen/ Fibrin 2:1:1, C) 3C=Fibrin/ Collagen/ Elastin 2:1:1, D) 3D=Fibrin /Collagen/ Elastin 1:1:1;Figure 10 shows wettability of elastin scaffolds at 0 seconds (A), 4 seconds (B), 9 seconds (C) and water contact angle measurement per second (D);

Figure 11 shows water contact angle measurements per second for elastin-based scaffolds;

Figure 12 shows an accelerated degradation profile of an elastin scaffold over a period of time;

Figure 13 shows accelerated degradation profiles of elastin-based composite scaffolds;

Figure 14 shows SEM images of elastin scaffolds (50x and 1000x) and pore % from 0-120+ µm;

Figure 15 shows pore size pattern for elastin-based composite scaffolds;

Figure 16 shows mechanical testing of anelastin scaffold: pre-test scaffold (A), post-test scaffold (B), stress distribution on the scaffold (C) and break strength of the elastin scaffold (D) (*** denotes the statistical significance of $p < 0.0001$);

Figure 17 shows mechanical properties for elastin-based composite scaffolds;

Figure 18 shows developing chorio-allantoic membrane (CAM) on an elastin scaffold on embryonic day (ED) 12 (A), total vascular area (B), the processed image for CAM analysis(C) and a number of bifurcation points (D);

Figure 19 shows Vascular area (%) for elastin-based composite scaffolds;

Figure 20 shows a gene expression profile of an elastin scaffold;

Figure 21 shows gene expression profiles for elastin-based composite scaffolds;

Figure 22 shows the difference in swelling ratio between Elastin/Collagen and Elastin/Fibrin scaffolds;

Figure 23 shows the difference in degradation profiles between Elastin/Collagen and Elastin/Fibrin scaffolds;

Figure 24 shows the microstructure of Elastin/Collagen and Elastin/Fibrin scaffolds using SEM;

Figure 25 shows the pore size of distribution of Elastin/Collagen and Elastin/Fibrin scaffolds;

Figure 26 shows the results of a live/dead assay for Elastin/Collagen and Elastin/Fibrin scaffolds; and

Figure 27 shows the vascular area for Elastin/Collagen and Elastin/Fibrin scaffolds at day 12.

Example 1 - Elastin scaffolds

Fabrication method and materials

Insoluble elastin powder was obtained from Sigma (the source of elastin was derived from bovine neck ligament) (Fig. 1A). 100mg of insoluble elastin powder was mixed with 1ml of 0.5M oxalic acid ($C_2H_2O_4$) (freshly prepared) at room temperature (Fig.1B).

To cross-link the protein, a homobifunctional cross-linking agent, 1% glutaraldehyde (GTA) (v/v), was added to the solution (Fig.1C). The solution was cast in a well of a 24 well plate and incubated at 37°C with 5% CO_2 for one hour (Fig.1C).

The mixture was frozen at -80°C overnight (Fig.1D) and lyophilised for 48 hours to form a scaffold (Fig.1E).

The fabricated scaffold was brought to room temperature and washed with 0.1M Glycine buffer at pH=10.4 with 2 washes of 15 minutes each and washed with tris-glycine buffer for 15 minutes. To remove excess of oxalic acid and unbound glutaraldehyde, scaffolds were

washed with 0.1% w/v sodium boro-hydride ($NaBH_4$) a reducing agent for approximately 8 hours on a shaker.

Subsequently, scaffolds were washed with distilled warm water (60^0 C) for 15 minutes and two washes of distilled water for 30 minutes each to remove remaining unbound glutaraldehyde from the scaffold.

For sterilisation, scaffolds were washed with 70% ethanol for 15 minutes and then with PBS.

Structural integrity and stability

The fabricated crosslinked elastin scaffold was intact (Fig. 2B). However, the non-crosslinked scaffold was dismantled/disintegrated (Fig. 2A).

An *in vitro* scaffold stabilisation study was carried out by comparing scaffolds with and without cross-linking for 28 days in PBS at 37°C and 5% CO_2. It was found that non-crosslinked scaffolds (Fig. 3A) were dismantled/disintegrated after 28 days in PBS and in contrast crosslinked scaffolds were intact (Fig. 3B). This indicates that this method of fabrication effectively produced an integral scaffold.

Biological activity

To evaluate the efficacy and biological activity of the scaffolds, adipose-derived stem cells (ADSCs) were cultured under standard culture conditions i.e. incubation at 37°C with 5% CO_2 in MesenPRO RS™ basal cell culture medium (ThermoFisher, UK) supplemented with 2% MesenPRO RS™ growth supplement (ThermoFisher, UK) and 1% penicillin/streptomycin (Sigma-Aldrich, UK). 50000 cells were seeded on 6mm diameter scaffolds and cultured for 1, 3 and 7 days. Cell survival and proliferation were studied using live/dead and alamar blue assays respectively. ADSCs were alive and adhered to the scaffold by day 1 and exhibited non-aggregated morphology on days 3 and 7 (Fig. 4). Additionally, cells maintained their non-aggregated behavior and demonstrated spindle morphological structure (Fig. 4) suggesting they retain their stem characteristics during the culture period.

Cell proliferation was quantitatively measured by alamar blue activity, a cell metabolic assay, and the absorbance at 570 nm was measured using a spectrophotometer at days 1, 3, and 7 (n=3 per time point) (Fig. 5).

Scanning Electron Microscopy

Elastin scaffolds were washed with distilled water in an ultra-sonic cleaner for 3 minutes to remove salts and dried for 24 hours in a lyophiliser. Scaffolds were mounted on stubs and sputter-coated with carbon under vacuum. All images were obtained using a secondary electron detector in a Philips XL 30 Field Emission SEM, operated at 5 kV and average working distance was 10 mm.

The SEM images in Figures 6A and 6B show that elastin scaffolds have an homogeneous structure and are porous in nature. Figure 6A is at 50x magnification and Figure 2 is at 250x magnification.

Discussion

This is a very cost-effective and time-efficient way to fabricate elastin scaffolds because, as of the priority date of this application, 5 mg of insoluble elastin from bovine neck ligament cost £69.70 GBP (E1625) whereas 1 mg of soluble α-elastin costs £272.50 GBP (E6527) from Sigma™ as the commercial supplier.

The live/dead assay results showed that cells maintained their spindle morphological structure which is one of the characteristics of ADSCs. Since ADSC have contact inhibition behavior (Majd et al., 2011) by using an elastin scaffold within the scope of the invention, the inventors were able to maintain contact inhibition behavior up to day 7 (figure 4). This cell morphology can maintain ADSCs phenotype and multipotent characteristics without undergoing any differentiation (Zhang and Kilian, 2013). An increase in the alamar blue absorbance is an indication of constant cell proliferation. These results also show that the fabricated scaffold was non-toxic to the cells.

Example 2 - Elastin/collagen/fibrin scaffolds

Fabrication method and materials

Tube 1: Elastin powder (9.7% w/v) + 0.5M oxalic acid + 3% glutaraldehyde (w/v).

Tube 2: Collagen hydrogel - prepared using 80% rat tail collagen type I (v/v) (First Link, Birmingham, UK) and 10% of 10X Minimal Essential Medium (Invitrogen, Paisley, UK), neutralised using 5M and 1M sodium hydroxide (Sigma-Aldrich, Dorset, UK) and added 10X DMEM.

Tube 3: Fibrin gel - prepared with 2% fibrinogen (w/v) dissolved in 1 ml of PBS and for fibrillogenesis, 1% thrombin (w/v) was added along with 0.1M CaCl2

Tubes 1 to 3 were mixed in varying ratios, cast and then incubated at 37°C with 5% CO_2 for 1 hour. The final volume after mixing the 3 tubes was always 1ml, which was then cast.

- For scaffolds that were 2:1:1 (collagen/elastin/fibrin), 500µl of Tube 2 were mixed with 250µl of Tube 1 and 250µl of Tube 3 (Also referred to herein as scaffold **3A**).
- For scaffolds that were 2:1:1 (elastin/collagen/fibrin), 500µl of Tube 1 was mixed with 250µl of Tube 2 and 250µl of Tube 3 (Also referred to herein as scaffold **3B**).
- For scaffolds that were 2:1:1 (fibrin/elastin/collagen), 500µl of Tube 3 were mixed with 250µl of Tube 1 and 250µl of Tube 2 (Also referred to herein as scaffold **3C**).
- For scaffolds that were 1:1:1, 333.3µl of each tube were mixed and cast (Also referred to herein as scaffold **3D**).

The mixture was freeze-dried for 48 hours.

Washing: First, a wash for 15 minutes with tris-glycine buffer. Second, to remove excess and unbound glutaraldehyde, scaffolds were washed with 0.1% sodium boro-hydride ($NaBH_4$) a reducing agent for approximately 8 hours on a shaker.

Biocompatibility

To evaluate biocompatibility of each combination scaffold, 50000 adipose derived stem cells (ADSC) were seeded per scaffold and cultured up to 7 days. Cell survival and proliferation at 1, 3 and 7 days after seeding were studied using live/dead and alamar blue assays respectively.

As an example, results for the three-component scaffolds show that ADSC were alive and adhered to the scaffold (Figure 7) and were proliferating until day 7 (Figure 8). The same results were observed in two-component scaffolds (i.e. scaffolds comprising elastin and collagen, or elastin and fibrin).

Microstructure

Microstructure of each scaffold was studied using SEM. Results for three-component scaffolds (Figure 9) showed that each scaffold combination has a unique ultrastructural

fibril network and pore size. Similar observations were made for two-component scaffolds. This variation in the structure could alter ADSC behaviour and differentiation as well as biomechanical properties of the scaffolds (See Ghasemi-Mobarakeh et al (2015)).

Example 3 - Water contact angle (WCA)

The wettability of the elastin scaffold was investigated by developing an experimental setup and a 30 µL distilled water droplet was dispensed onto each scaffold and several images were taken over the time interval between 0 to 5 seconds. The time at 0 seconds was considered the initial time of contact with a liquid medium (water). The WCA was calculated using Young's equation and the angle was measured from the water-scaffold interface to the line tangent and perimeter of the water droplet (Fu et al (2014)). The calculated WCA is a demonstration of water-material interaction.

The calculated WCA for elastin at 0 seconds was 102 ± 7.75° and it was reduced to 73.88 ± 5.90° at 4 seconds. Over the time WCA continued to decrease over time and at reached 0° at 9 seconds which indicated complete wettability of the elastin scaffold (Figure 10).

However, by combining elastin with other natural polymers such as fibrin and collagen at different ratios the WCA for 3A (68.18 ± 3.38° at 0 seconds to 0° at 3 seconds), 3C (67.46 ± 4.51° at 0 seconds to 0° at 4 seconds) was altered and showed complete wettability by 4 seconds. Interestingly WCA for 3B (112.34 ± 5.37° at 0 seconds to 99.32 ± 14.55° at 10 seconds) and 3D (120.18 ± 5.36° at 0 seconds to 113.23 ± 8.93 at 10 seconds) (Figure 11) did not show complete wettability even at 10 seconds making them hydrophobic as for any material > 90° is considered to be hydrophobic therefore elastin, 3A and 3C scaffolds demonstrated hydrophilic nature and showed high cohesion towards water and gained complete wettability by 9 seconds. but scaffolds 3B and 3D showed to hydrophilic nature with low cohesion towards the water.

Example 4 - Accelerated Trypsin Degradation

To measure the stability of scaffolds, an accelerated degradation profile was carried out by using 1X trypsin. An initial weight of scaffolds was measured using XS205 *Mettler Toledo*® digital scale. The scaffolds were placed in 24 well-plate with 1X trypsin and incubated at 37 °C and with 5% CO_2. At each time point, scaffolds were washed with distilled water and lyophilised and weight was measured.

A net change in the weight was measured as a parameter of the degradation. In vitro accelerated degradation results indicated that elastin scaffold degraded from day 1 (136.06 ± 11.90 mg). By the day 5, there was 25% decrease in the weight and this trend continued and by day 42 there was 70% degradation of the scaffold (Figure 12).

The degradation profile for the elastin-based co-polymers was identical for 3A,3C and 3D. By day 7 almost 40% scaffolds were degraded this pattern was continued until day 42 where almost 70% of scaffolds were degraded. However, 3B, which has 50% of elastin, was the most stable scaffold with 55% degradation until day 42 (Figure 13). This shows that different degradation pattern of elastin-based scaffolds can be used for various tissue engineering application depending upon regenerative properties of each tissue type.

Example 5 – Structural Properties

To measure pore size range and porosity, all SEM images were quantitively analysed using ImageJ bundled with 64-bit Java 1.6.0 (NIH, USA). A threshold function was used to visualise all pores in the scaffold. Additionally, friction area, particle analysis function was used.

Calculated pore size percentages for the scaffolds were in the range of 0-120+ µm and 28% pores were in the range of 0-19 µm, 48% pores in the range of 20-79 µm and remaining 24% in the range of 80- 120+ µm (Figure 14) and total porosity of scaffold was 48%.

When elastin was combined with other polymers, 70% pores were present in the 0-59 and remaining 50% in the range of 60-120+ µm in 3A. In 3B, the majority of pores (65%) were in the range of 20-59 µm but in 3C pore pattern was uniform and 55% pores were in the range of 20-59 µm. However, in 3D 75% pores were in the 0-59 µm range (Figure 15).

Pore and porosity play a vital role in the angiogenesis and diffusion of nutrients. The results suggest that elastin-based scaffolds could be used for various tissue engineering applications.

Example 6 - Mechanical properties

The elastin scaffold was tested to failure using bi-axial BioTester (CellScale Biomaterials Testing, Canada). The system includes 2 high-performance actuators with temperature-controlled media bath to avoid scaffold drying while testing cell-seeded scaffolds. To analyse real-time stress distribution, a time synchronised high-resolution CCD camera for the acquisition and processing of the test results was used.

The wet mechanical properties of elastin scaffold at day 0 was 154 ± 1 mN and after seeding hADSC cells for 28 days the strength of the scaffold significantly ($p < 0.0001$) increased to 185.5 ± 1.5 mN (Figure 16). This demonstrates that cells seeded in the scaffolds add to the mechanical integrity of scaffold by tissue remodelling mechanism.

The calculated break strength for the 3A 74.33±3.78 mN, 119.33±33.12mN for 3B, 103.34±20.23mN for 3C and 71.68±4.72mN for 3D. This demonstrates that after adding another co-polymer the mechanical properties of elastin decrease. It is believed that this is due to the non-fibril arrangement of the polymers (Lake et al. (2012)).

Example 7 - Angiogenesis

Pathogen-free fertilised eggs were obtained from a commercial supplier and incubated for 3 days at 38°C with 40-45% humidity. On an embryonic day (ED), 3 (ED 3) *ex ovo* glass bowl set-up was constructed to grow the embryonic culture and maintained at 37.5°C with 3% CO_2 and an average humidity in the range of 80-85% (3). At ED 9 elastin scaffold were placed on the developing chorio-allantoic membrane (CAM) to allow infiltration of blood vessels and at ED 12 embryos were euthanised as per home office guideline, and scaffolds were excised and fixed in 4% glutaraldehyde and analysed.

A total calculated vascular area for ED 10 was 4.78 ± 2.12% and this vascular area increased to 6.01 ± 3.34% at ED 11 although this increment was not statistically significant but developed two large vessels with a number of capillary plexus. This trend continues to follow on ED 12 with the calculated vascular area was 8.34 ± 2.67% (Figure 18).

When elastin was combined with fibrin and collagen, in different ratios, then there was an increase in the total vascular area % by day 12. The calculated % vascular area was 12.97 ± 0.61% for 3A, 11.33±1.52% for 3B, 14.41±0.67% for 3C and 16.52 ±0.57 for 3D (Figure 19). Therefore, it appears beneficial to use a combination of polymers to enhance angiogenetic properties of elastin. A CAM assay acted as ex vivo bioreactor to understand

vascular invasion into the elastin-based scaffolds. In view that scaffolds have pore distribution in the range of 0 µm - 120+ µm which act as a pro-angiogenetic material for blood vessels infiltration.

Example 8 - Cellular differentiation

To understand human adipose-derived cells (hADSCs) differentiation pathway on the elastin scaffold, a total 5X 10^5/mm^3 hADSC of passage 4 seeded on scaffolds. RNA was isolated by using TRIzol (Invitrogen, Paisley, UK) method on day 1,7 14 and total RNA yield was quantified by using spectrophotometer (Spectronic Camspec Ltd, Garforth, UK). cDNA synthesis was carried out using Precision nanoscript 2 reverse transcription kit (Primer Design, Southampton, UK) and quantitative PCR was performed with custom designed and synthesised primers (Table 1) (Primer Design, Southampton, UK).

Table 1 Forward and reverse primers

Name of gene	Forward primer	Reverse primer
MYOD1	CGCCTGAGCAAAGTAAATGAG (SEQ ID: 1)	GCCCTCGATATAGCGGATG (SEQ ID: 2)
PPARG	GAATAAAGATGGGGTTCTCATATCC (SEQ ID: 3)	AACTTCAGCAAACTCAAACTT (SEQ ID: 4)
CEBPA	CGGCAACTCTAGTATTTAGGATAAC (SEQ ID: 5)	CAAATAAAATGACAAGGCACGATT (SEQ ID: 6)
RUNX2	TTCTCCCCTTTTCCCACTGA (SEQ ID: 7)	CAAACGCAATCACTATCTATACCAT (SEQ ID: 8)
SOX9	GGACCAGTACCCGCACTTG (SEQ ID: 9)	AATCCGGGTGGTCCTTCTTG (SEQ ID: 10)
OCT4	CACTAAGGAAGGAATTGGGAACA (SEQ ID: 11)	GGGATTAAAATCAAGAGCATCATTG (SEQ ID: 12)
REX1	CGTTTCGTGTCCCTTTCA (SEQ ID: 13)	CCTCTTGTTCATTCTTGTTCGTATT (SEQ ID: 14)

Gene expression of and mesenchymal lineage-specific differentiation markers Adipogenic (CEBPA and PPARG), Osteogenic (RUNX2), Myogenic (MYOD1), Chondrogenic (SOX9) and MSC markers (OCT4 and REX1) were studied in hADSCs.

Differentiation profile of hADSC on the elastin scaffold. OCT4, CEBPA, PPARG and MYOD1 showed an identical trend of significant upregulation by 0.03-0.04 units on day 7 and 14 in comparison to day 1 ($p<0.0001$). However, there was no significant upregulation on day 14 in comparison to day 7. RUNX2 did not show any trend. SOX9 exhibited negligible expression (<0.027) at all three-time points identical to all the other scaffolds

reported above, although it showed a significant upregulation on day 14 (0.025, $p<0.05$) in comparison to day 1 (0.027). REX1 exhibited an initial downregulation on day 7 (0.036 to 0.028, $p<0.0001$), followed by a significant upregulation trend on day 14 (0.031, $p<0.0001$) (Figure 20).

In 3A , Oct-4 shows significant downregulation on day 7 and 14 (0.028, $p< 0.0001$) from day 1 (0.031).Rex-1 downregulated significantly on day 7 (0.026, $p< 0.0001$)and 14 (0.029, $p< 0.0001$) in comparison to day 1 (0.031).However, expression on day 14 was significantly higher than day 7 ($p< 0.0001$), whereas MyoD-1 was constant at 0.032. CEBP showed a marginal upregulation on day 7 ($p< 0.05$) and significantly downregulated to 0.025 on day 14 ($p< 0.0001$). In 3B, Oct-4, RUX-2 and CEBP showed significant downregulation on day 7 and 14 ($p< 0.0001$) in comparison and there was no significant difference between expression on day 7 and 14 . In 3C, Oct-4 showed a steady and significant downregulation from 0.030 on day to 0.029 on day 14 ($p<0.001$). Rex-1 and RUNX-2 downregulated significantly ($p<0.0001$) from 0.032 on day 1 to 0.030 and 0.029 respectively on day 7. In 3D, Oct-4, CEBP, PPAR-gamma and MyoD-1 showed identical trend of significant downregulation by 0.04-0.06 units on day 7 and 14 in comparison to day 1 ($p<0.0001$)

Example 9 - Binary elastin-based scaffolds

- Elastin/Collagen – 1:1 ratio
- Elastin/Fibrin – 1:1 ratio

The elastin, collagen and fibrin were prepared as shown in Example 2.

- **Swelling ratio**

Figure 22 shows the difference in swelling ratio between Elastin/Collagen and Elastin/Fibrin scaffolds. Swelling ratio is an indication of the interaction between a solvent and a polymer. It shows exchange of the affinity and enthalpy between two phases. The higher the crosslinking density inside a polymer then the lower the swelling property, and vice-versa. The swelling ratio (SR)of the elastin and its composites was measured from dry mass and wet mass with the following equation

$$SR = \frac{M_w - M_d}{M_w} \qquad (1)$$

where M_d is the dry weight of the scaffold and M_w is the wet weight of the scaffold. A wet mass of the scaffold was measured by immersing into 2ml of distilled water. Dry and wet mass measured with the digital scale (XS205 *Mettler Toledo*®) and the SR was calculated using equation (1)

- **Degradation**

Figure 23 shows the difference in degradation profiles between Elastin/Collagen and Elastin/Fibrin scaffolds. The experimental protocol was the same as described in Example 4.

- **Microstructure**

Figure 24 shows the microstructure of Elastin/Collagen (A) and Elastin/Fibrin (B) scaffolds using SEM.

- **Pore size distribution**

Figure 25 shows the pore size of distribution of Elastin/Collagen and Elastin/Fibrin scaffolds.

- **Biological activity**

Figure 26 shows the results of a live/dead assay for Elastin/Collagen and Elastin/Fibrin scaffolds. The experimental protocol was the same as described in Example 1.

- **Angiogenesis**

Figure 27 shows the vascular area for Elastin/Collagen and Elastin/Fibrin scaffolds at day 12. The experimental protocol was the same as described in Example 7.

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Claims

1. A method for forming a tissue scaffold, comprising cross-linking a composition, the composition comprising elastin, wherein the elastin is unfractionated and comprises solubilised elastin.

2. A method according to claim 1, comprising a step of solubilising elastin.

3. A method for forming a tissue scaffold, comprising cross-linking a composition comprising unfractionated solubilised elastin.

4. A method according to claim 3, comprising a step of solubilising elastin to form the composition comprising unfractionated solubilised elastin.

5. A method according to any preceding claim, wherein the elastin is, or has been, solubilised by contacting with oxalic acid.

6. A method according to any preceding claim, wherein the elastin is, or has been, solubilised at a temperature less than 100°C.

7. A method according to claim 6, wherein the step of solubilising the elastin is, or has been, carried out at a temperature less than or equal to 50°C.

8. A method according to claim 7, wherein the step of solubilising the elastin is , or has been, carried out at a temperature of 15 to 30°C.

9. A method according to any preceding claim, wherein the composition that is cross-linked comprises insoluble elastin.

10. A method for forming a tissue scaffold, comprising cross-linking a composition, the composition comprising soluble elastin and insoluble elastin.

11. A method according to any preceding claim, wherein the composition that is cross-linked comprises collagen and/or fibrin.

12. A method according to claim 3 or claim 4, or any claim dependent on claim 3 or claim 4, comprising cross-linking a composition comprising unfractionated solubilised elastin and a) collagen and/or b) fibrin.

13. A method according to any of claims 11 to 12, wherein the collagen is in the form of a collagen hydrogel.

14. A method according to any of claims 11 to 13, wherein the fibrin is in the form of a fibrin gel.

15. A method according to any preceding claim, wherein the cross-linking comprises chemical cross-linking.

16. A method according to claim 15, wherein the cross-linking comprises contacting the composition with an aldehyde cross-linking agent.

17. A method according to claim 16, wherein the aldehyde cross-linking agent is glutaraldehyde.

18. A method according to any preceding claim, wherein the cross-linking is carried out at a temperature of 25°C to 50°C.

19. A method according to any preceding claim, wherein the cross-linking takes place in the presence of CO_2.

20. A method according to claim 19, wherein the cross-linking takes place in the presence of 2 to 10% CO_2.

21. A method according to any preceding claim, wherein the cross-linking takes place from 1 to 24 hours.

22. A method according to claim 3 or claim 4, or any claim dependent on claim 3 or claim 4, comprising lyophilising the composition comprising cross-linked unfractionated solubilised elastin.

23. A method according to any preceding claim, wherein lyophilisation takes place following solubilisation and cross-linking.

24. A method of forming a tissue scaffold comprising lyophilising a composition comprising cross-linked elastin, optionally wherein the composition comprising cross-liked elastin has been formed by cross-linking a formulation comprising elastin that is unfractionated and comprises solubilised elastin.

25. A method according to claim 24, comprising lyophilising a composition comprising cross-linked, unfractionated, solubilised elastin.

26. A method according to any preceding claim, comprising washing to remove agents involved in solubilising and/or cross-linking.

27. A method according to claim 26 comprising lyophilisation and washing, wherein the washing takes place following lyophilisation.

28. A method according to claim 26 or claim 27, wherein the step of washing to remove agents involved in solubilising and/or cross-linking comprises washing with a reducing agent.

29. A method according to claim 28, wherein the reducing agent comprises sodium borohydride or agents with similar carbonyl group reactivity.

30. A method according to any of claims 26 to 29, wherein the step of washing to remove agents involved in solubilising and/or cross-linking is carried out for at least 5 hours, preferably at least 8 hours.

31. A method according to any preceding claim comprising sterilising the scaffold.

32. A method according to claim 31, comprising contacting the scaffold with ethanol.

33. A composition comprising a) elastin, a solubilising agent that is able to solubilise the elastin and a cross-linking agent, or b) elastin, and a cross-linking agent, wherein the elastin in the composition is unfractionated and comprises solubilised elastin.

34. A composition comprising unfractionated, solubilised elastin and a cross linking agent.

35. A composition according to claim 33 or claim 34 comprising collagen and/or fibrin.

36. A tissue scaffold obtained or obtainable by a method as disclosed in any of claims 1 to 32.

37. A tissue scaffold comprising cross-linked elastin, wherein the cross-linked elastin has been formed by cross-linking a composition comprising elastin that is unfractionated and comprises solubilised elastin.

38. A tissue scaffold comprising cross-linked unfractionated solubilised elastin.

39. A tissue scaffold comprising cross-linked elastin, wherein the cross-linked elastin has been formed by cross-linking a composition comprising soluble elastin and insoluble elastin

40. A tissue scaffold according to claim 37 wherein the cross-linked elastin has been formed by cross-linking a composition comprising insoluble elastin, or according to claim 38, wherein the tissue scaffold comprises insoluble elastin.

41. A tissue scaffold according to any of claims 37 to 40, comprising collagen and/or fibrin.

42. A tissue scaffold according to any of claims 37 to 41, which is lyophilised.

43. A tissue scaffold according to any of claims 37 to 44 which is sterile.

44. A method for promoting tissue regeneration, tissue healing or tissue repair comprising applying a tissue scaffold according to any of claims 36 to 43, to a patient in need thereof.

45. A method according to claim 44, wherein the method is for promoting soft tissue regeneration or repair.

46. A tissue scaffold according to any of claims 36 to 43, for use in promoting tissue regeneration, tissue healing or tissue repair.

47. The tissue scaffold for use according to claim 46, for promoting soft tissue repair.

48. A method for solubilising elastin, comprising contacting elastin with oxalic acid at a temperature less than 100°C.

49. A method according to claim 48, comprising contacting elastin with oxalic acid at a temperature less than 50 °C, preferably at a temperature of 15 to 30°C.

50. A method according to claim 48 or claim 49, wherein the elastin is contacted with the acid for 5 minutes or less, preferably from 1 to 3 minutes.

51. A method for solubilising elastin comprising contacting elastin with oxalic acid for 5 minutes or less.

52. A method according to claim 51, wherein the elastin is contacted with oxalic acid at a temperature less than 100°C.

53. A method according to claim 52, comprising contacting elastin with oxalic acid at a temperature less than 50 °C, preferably at a temperature of 15 to 30°C.

54. A method according to any of claims 48 to 53, wherein the oxalic acid is 0.2M to 1M.

55. A composition obtained or obtainable from a method according to any of claims 48 to 54.

56. A method comprising cross-linking a composition as defined in claim 55.

57. A tissue scaffold comprising i) elastin; and ii) collagen and/or fibrin, wherein the elastin is cross-linked.

58. A tissue scaffold according to claim 56, wherein the collagen and/or fibrin is cross-linked.

59. A tissue scaffold according to claim 57 or claim 58, wherein the elastin comprises solubilised elastin.

60. A tissue scaffold according to any of claims 57 to 59, wherein the elastin comprises insoluble elastin.

61. A tissue scaffold according to any of claims 57 to 60, wherein the elastin is unfractionated.

62. A composition comprising i) elastin, ii) a cross-linking agent; and iii) fibrin and/or collagen.

63. A composition according to claim 61, wherein the elastin comprises solubilised elastin.

64. A composition according to any of claims 62 to 63, wherein the elastin is unfractionated elastin.

65. A composition according to any of claims 62 to 64, comprising a solubilising agent for solubilising elastin.

66. A method comprising cross-linking a composition comprising i) elastin, and ii) collagen and/or fibrin.

67. A method according to claim 66, wherein the elastin is unfractionated.

68. A method according to claim 66 or claim 67, wherein the elastin comprises solubilised elastin.

69. A method according to any of claims 66 to 68, wherein the elastin comprises insoluble elastin.

70. A tissue scaffold according to any of claims 57 to 60 for use in promoting tissue healing, regeneration or repair.

71. A method for promoting tissue regeneration, tissue healing or tissue repair comprising applying a tissue scaffold according to any of claims 57 to 61, to a patient in need thereof.

72. A tissue scaffold according to any of claims 36 to 43 or 57 to 61, seeded with cells.

73. A method comprising seeding a scaffold with cells, wherein the scaffold is as defined in any of claims 36 to 43 or 57 to 61.

74. A cell or tissue culture comprising a scaffold, wherein the scaffold is as defined in any of claims 36 to 43 or 57 to 61

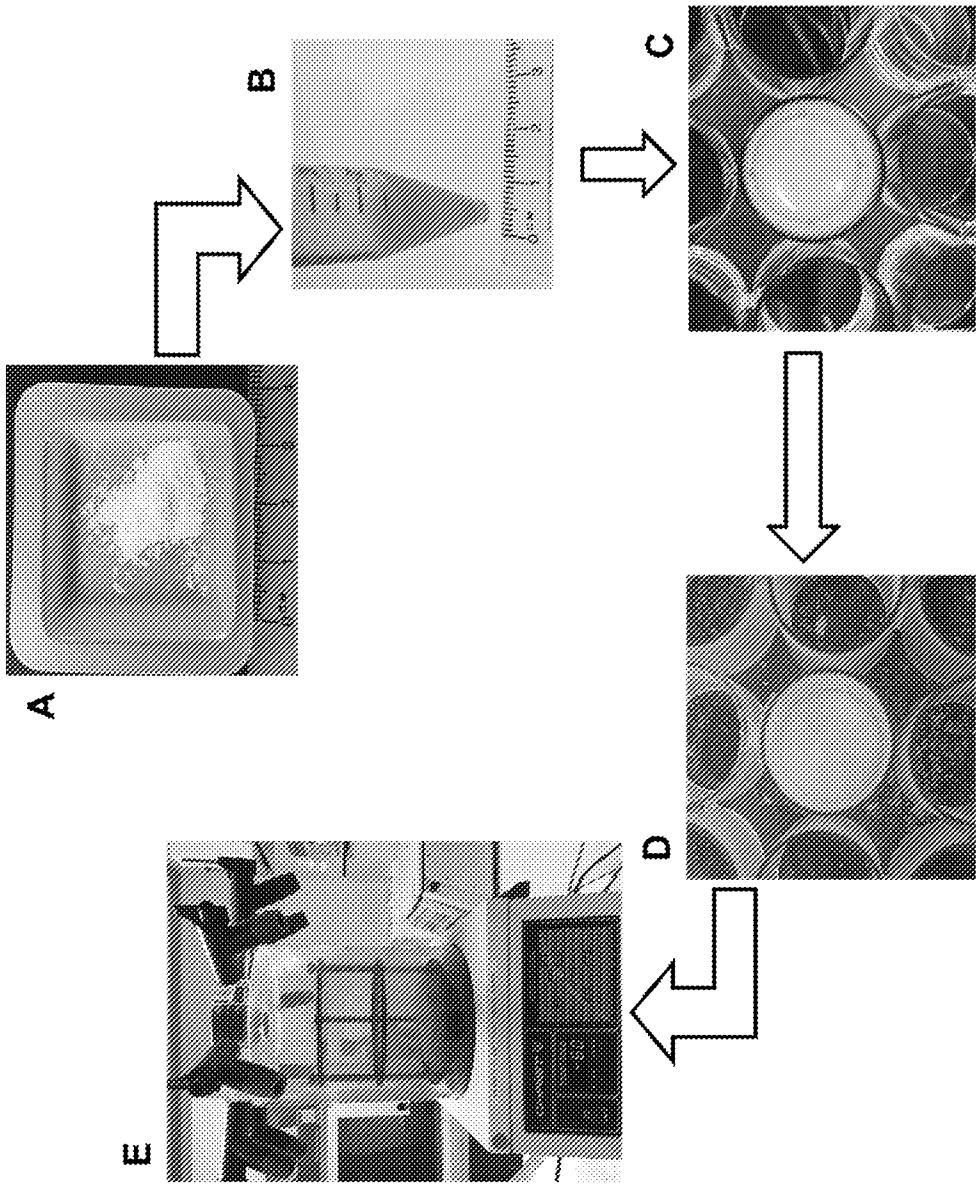


Figure 1

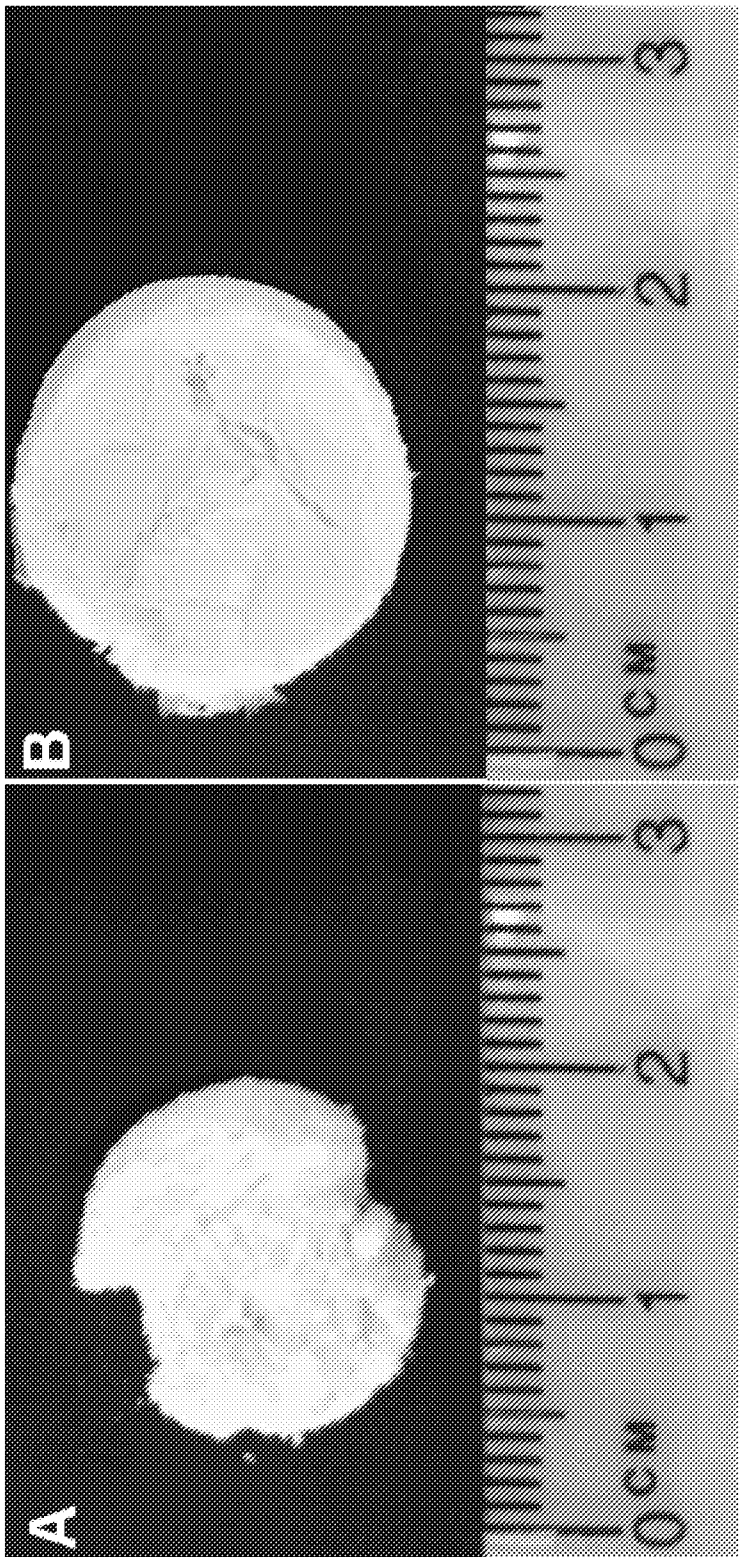


Figure 2

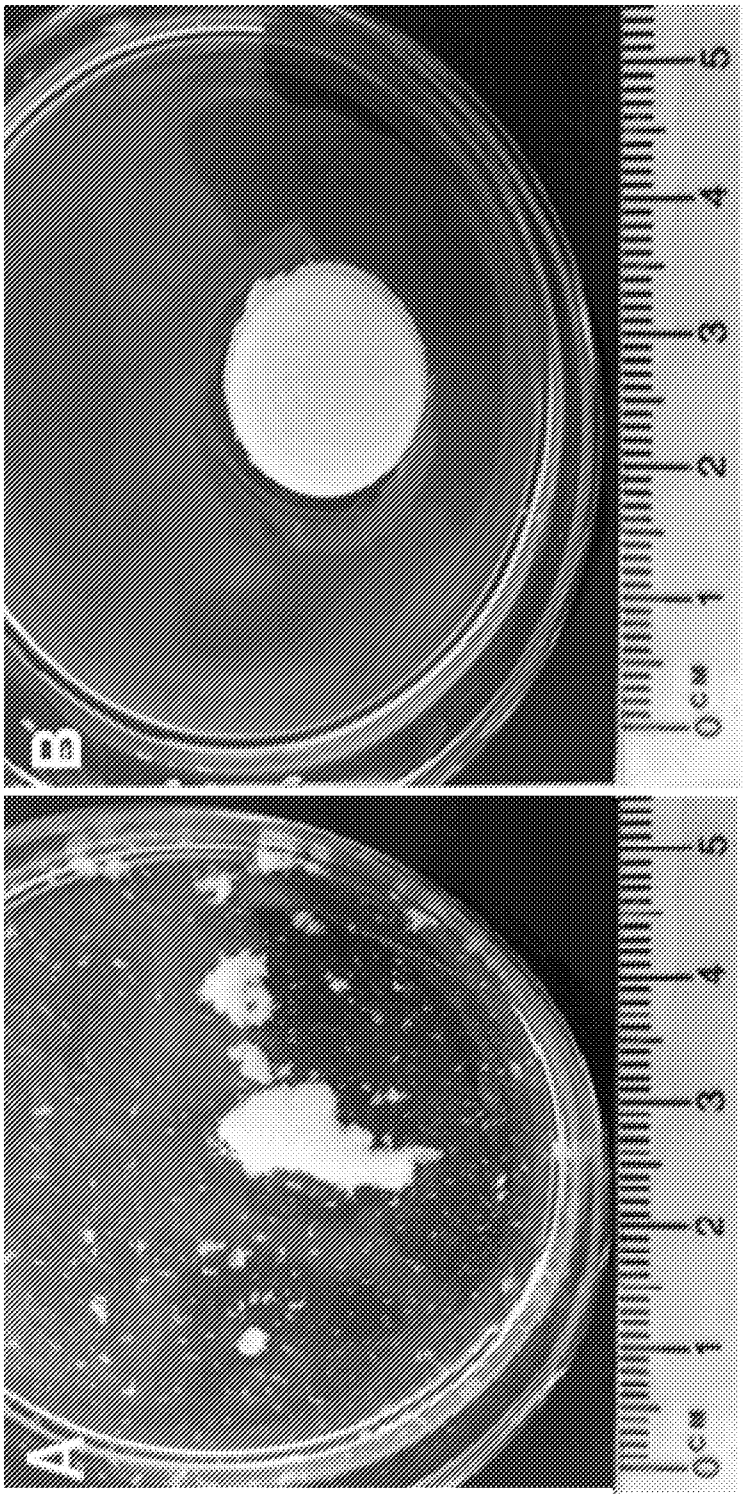


Figure 3

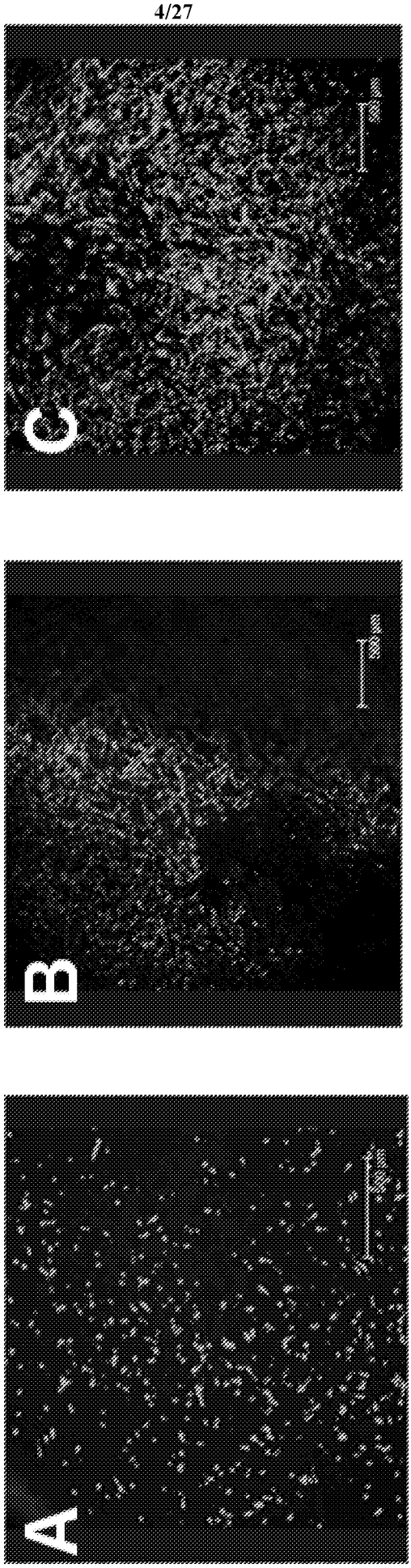


Figure 4

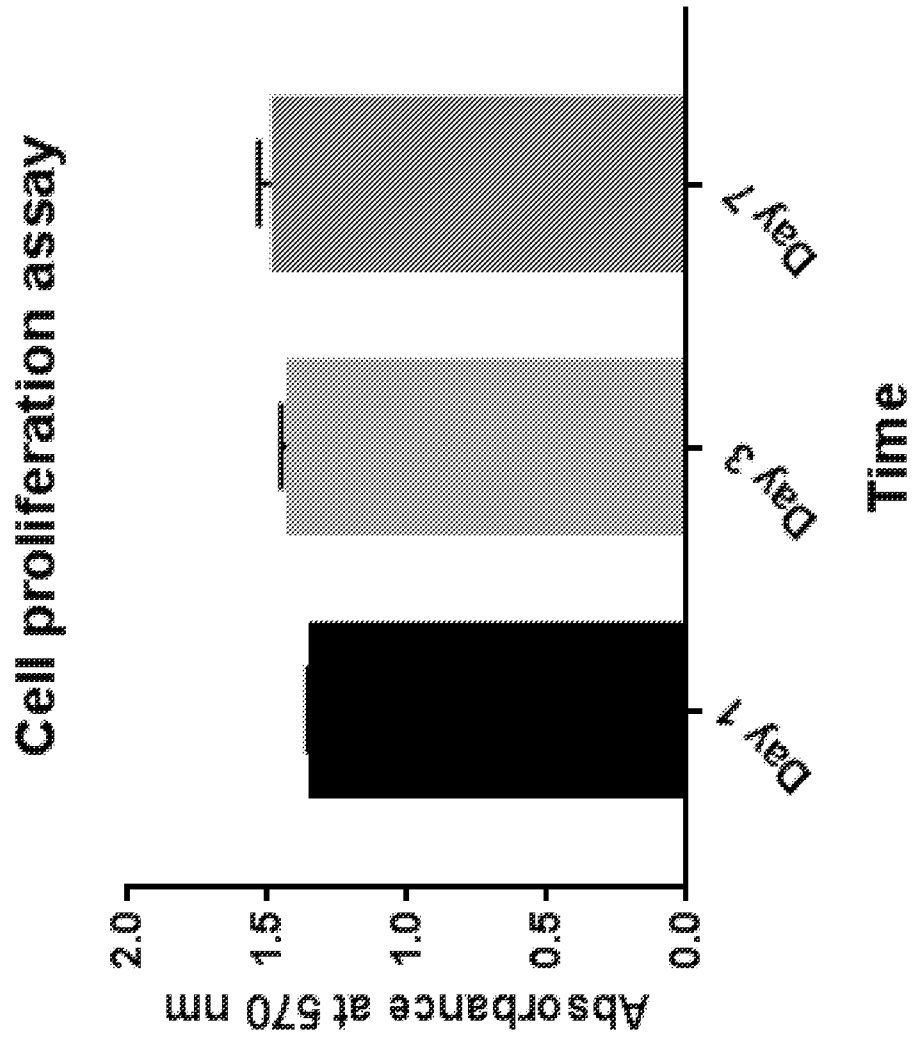
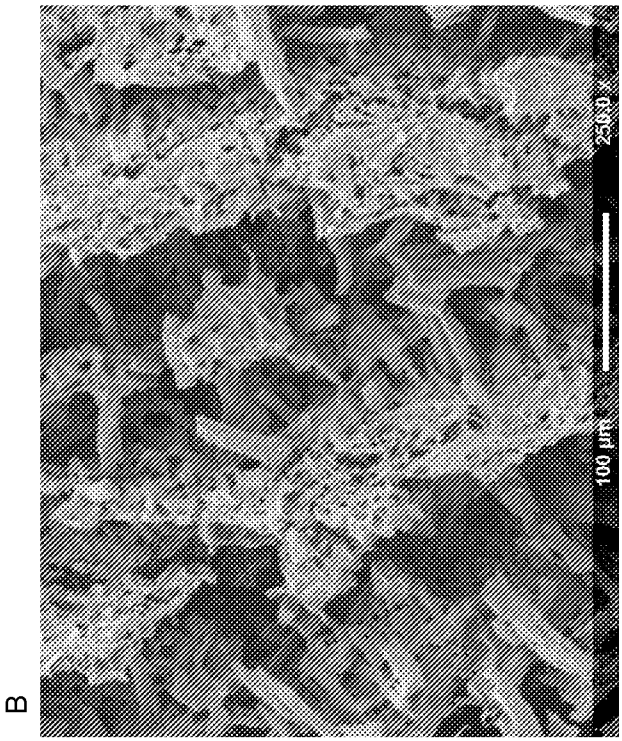

Cell proliferation assay
Absorbance at 570 nm
2.0
1.5
1.0
0.5
0.0
Day 1
Day 3
Day 7
Time

Figure 5



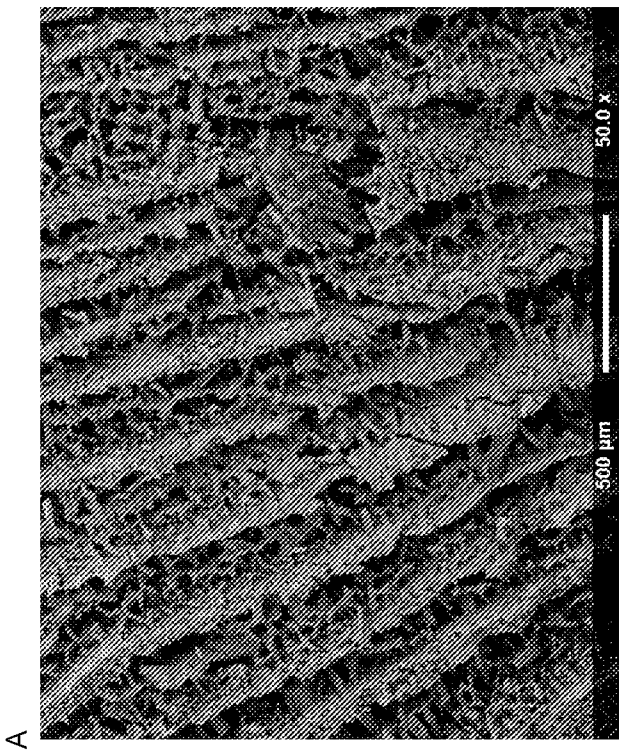


Figure 6

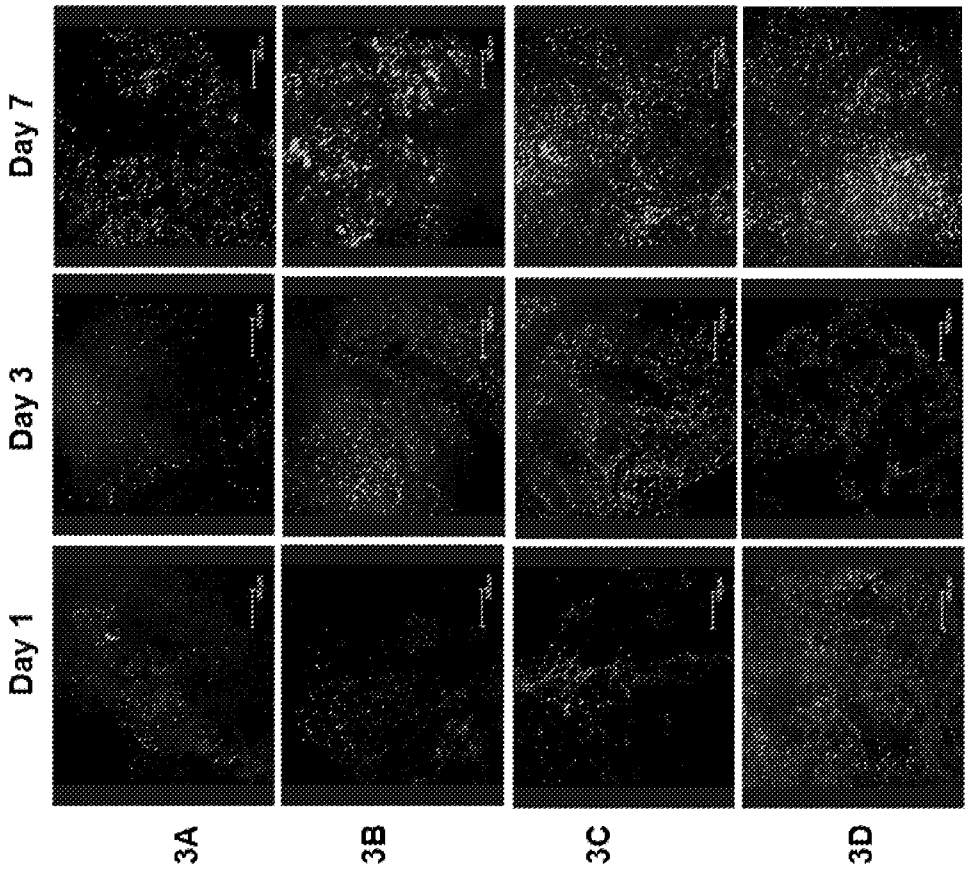


Figure 7

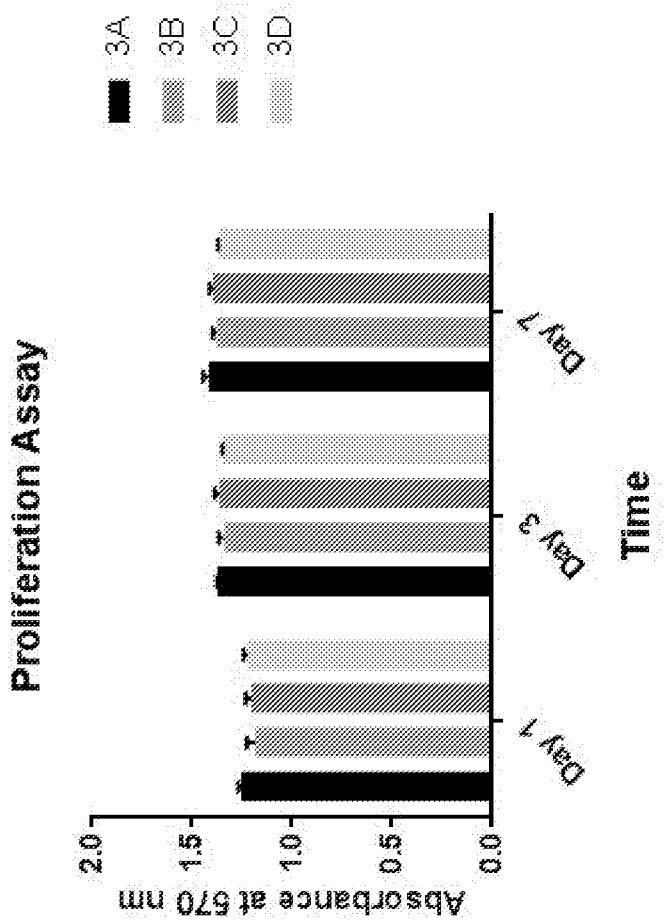

3A
3B
3C
3D
Proliferation Assay
Absorbance at 570 nm
2.0
1.5
1.0
0.5
0.0
Day 1
Day 3
Day 7
Time

Figure 8

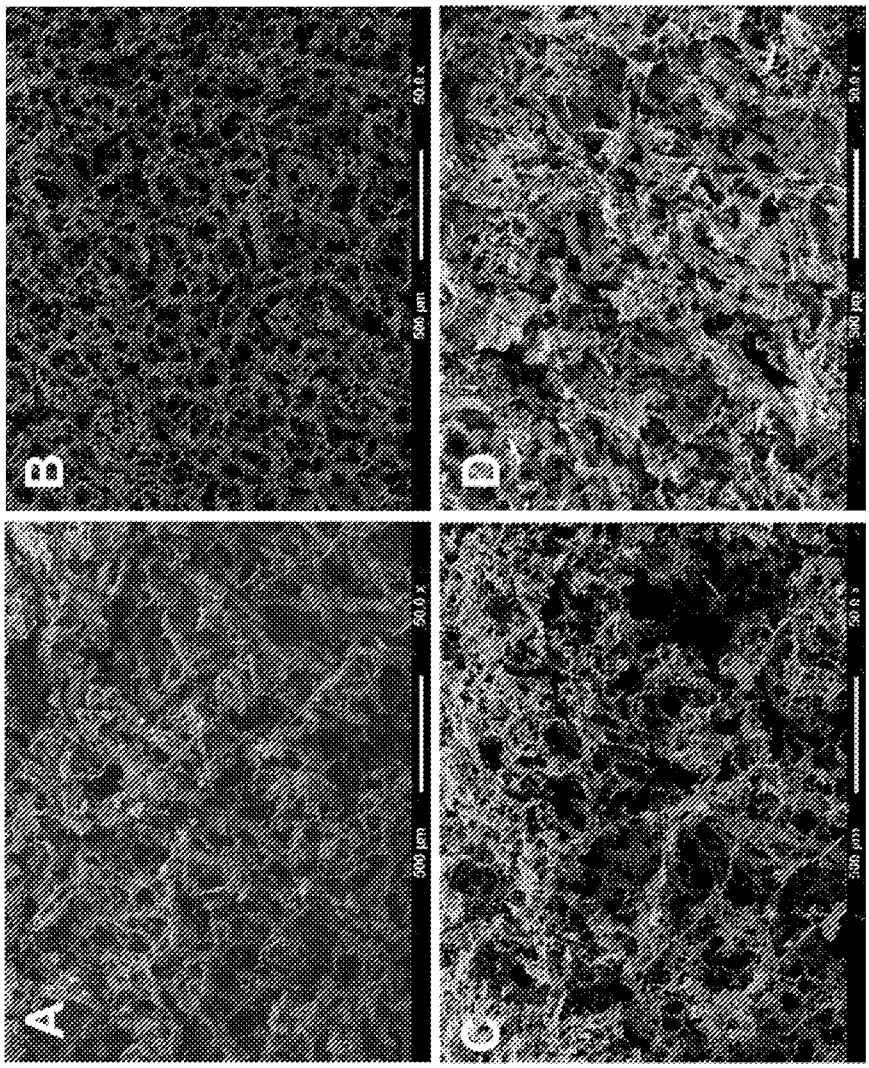


Figure 9

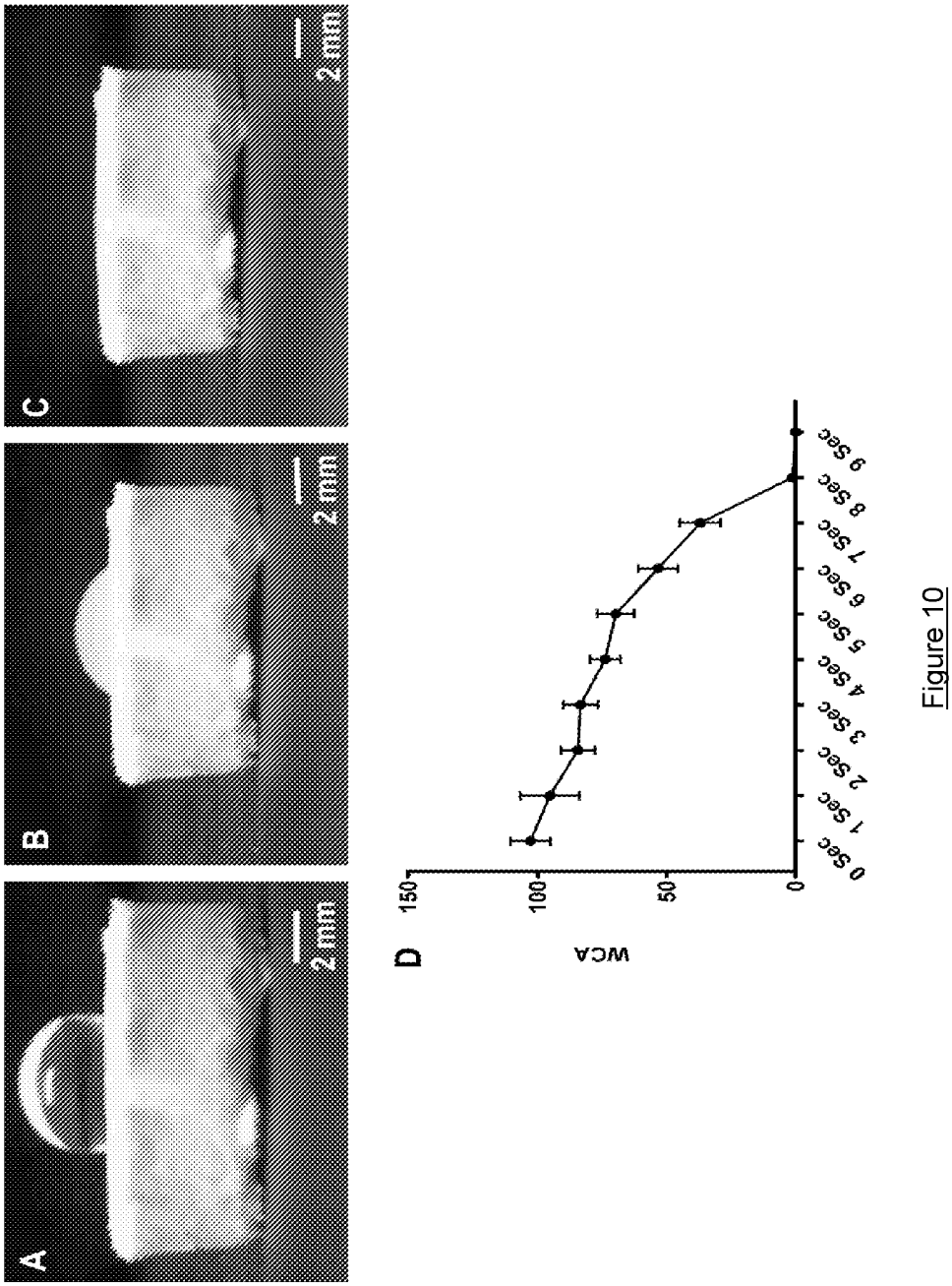

A
2 mm
B
2 mm
C
2 mm
D
WCA
150
100
50
0
0 Sec
1 Sec
2 Sec
3 Sec
4 Sec
5 Sec
6 Sec
7 Sec
8 Sec
9 Sec

Figure 10

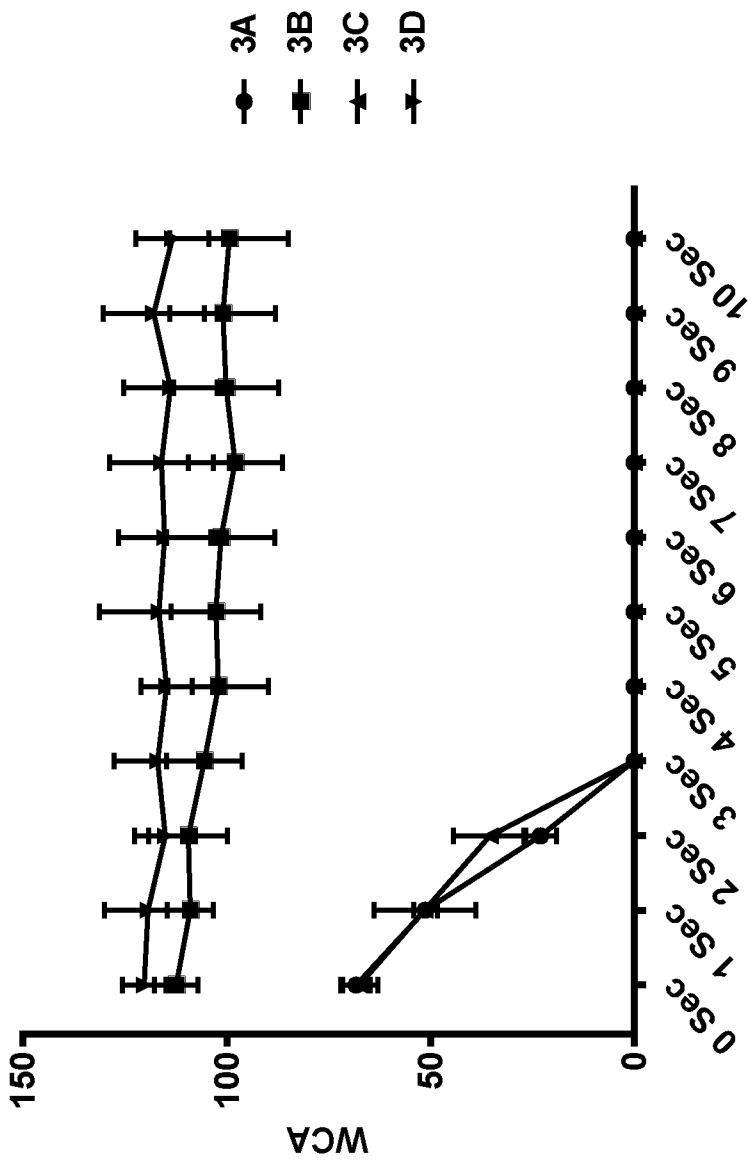

3A
3B
3C
3D
WCA
150
100
50
0
0 Sec
1 Sec
2 Sec
3 Sec
4 Sec
5 Sec
6 Sec
7 Sec
8 Sec
9 Sec
10 Sec

Figure 11

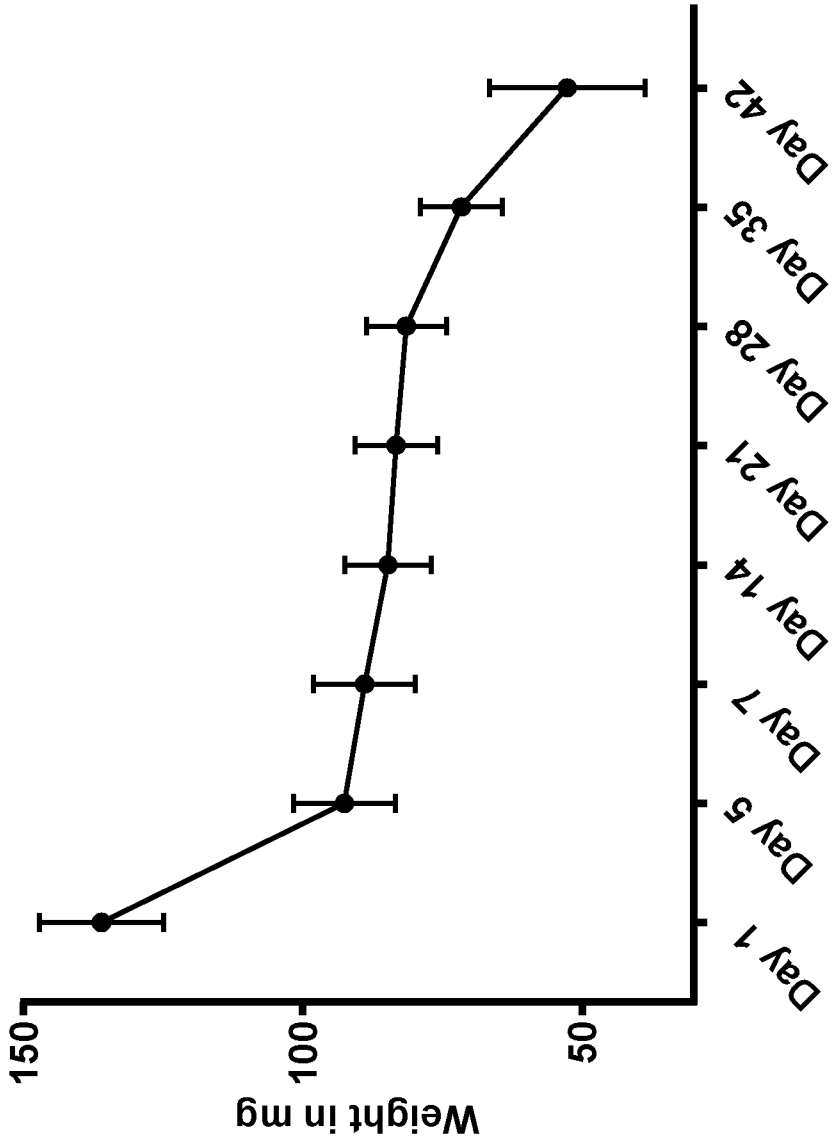

Weight in mg
150
100
50
Day 1
Day 5
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42

Figure 12

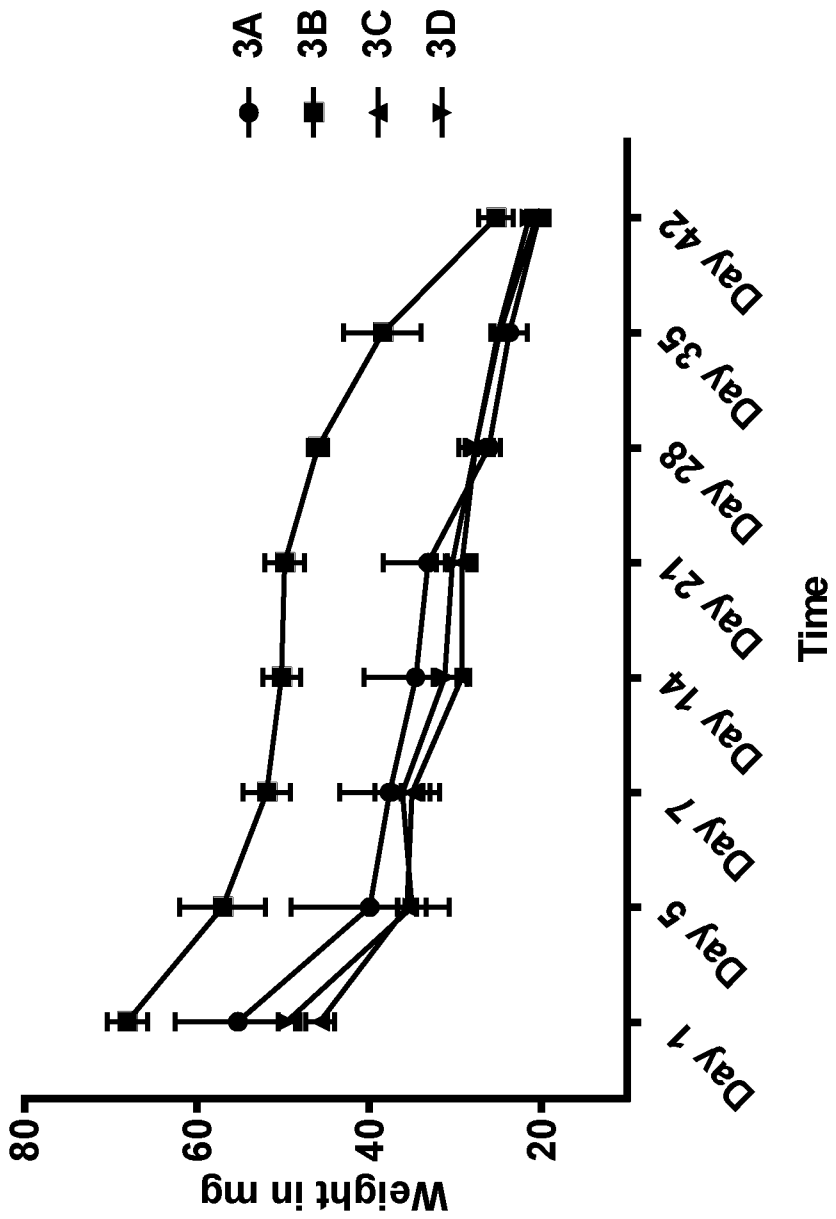
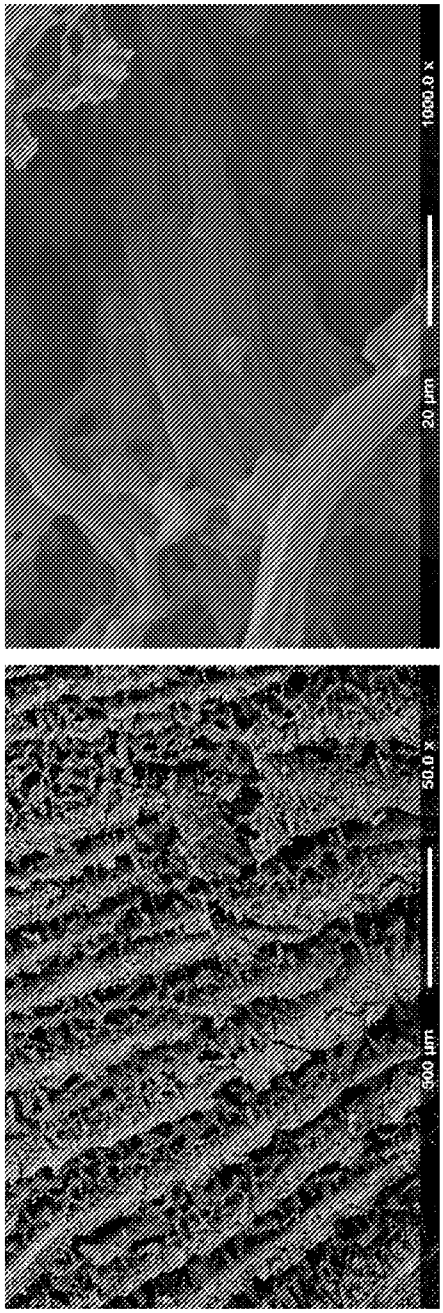

3A
3B
3C
3D
Weight in mg
80
60
40
20
Day 1
Day 5
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
Time

Figure 13


1000.0 x
20 µm
50.0 x
500 µm

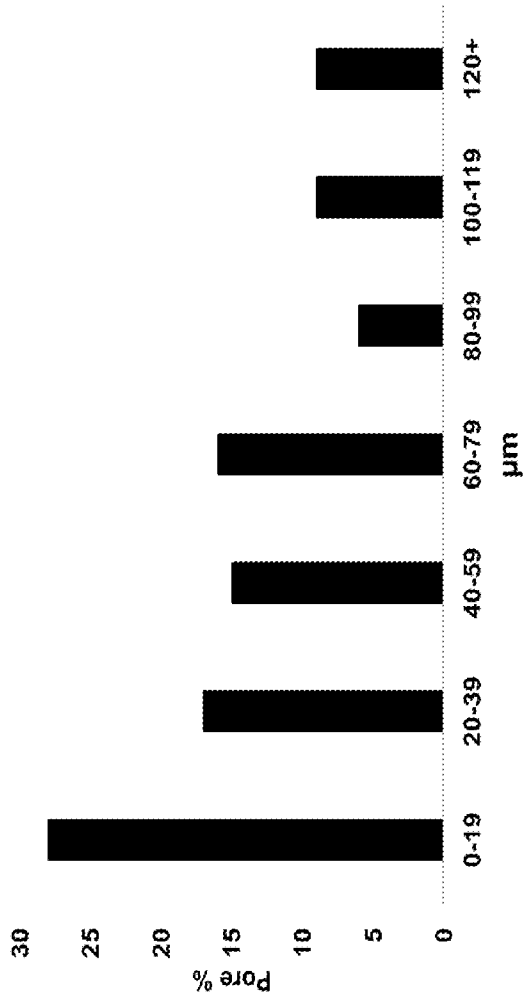

Pore %
30
25
20
15
10
5
0
0-19
20-39
40-59
60-79
80-99
100-119
120+
µm

Figure 14

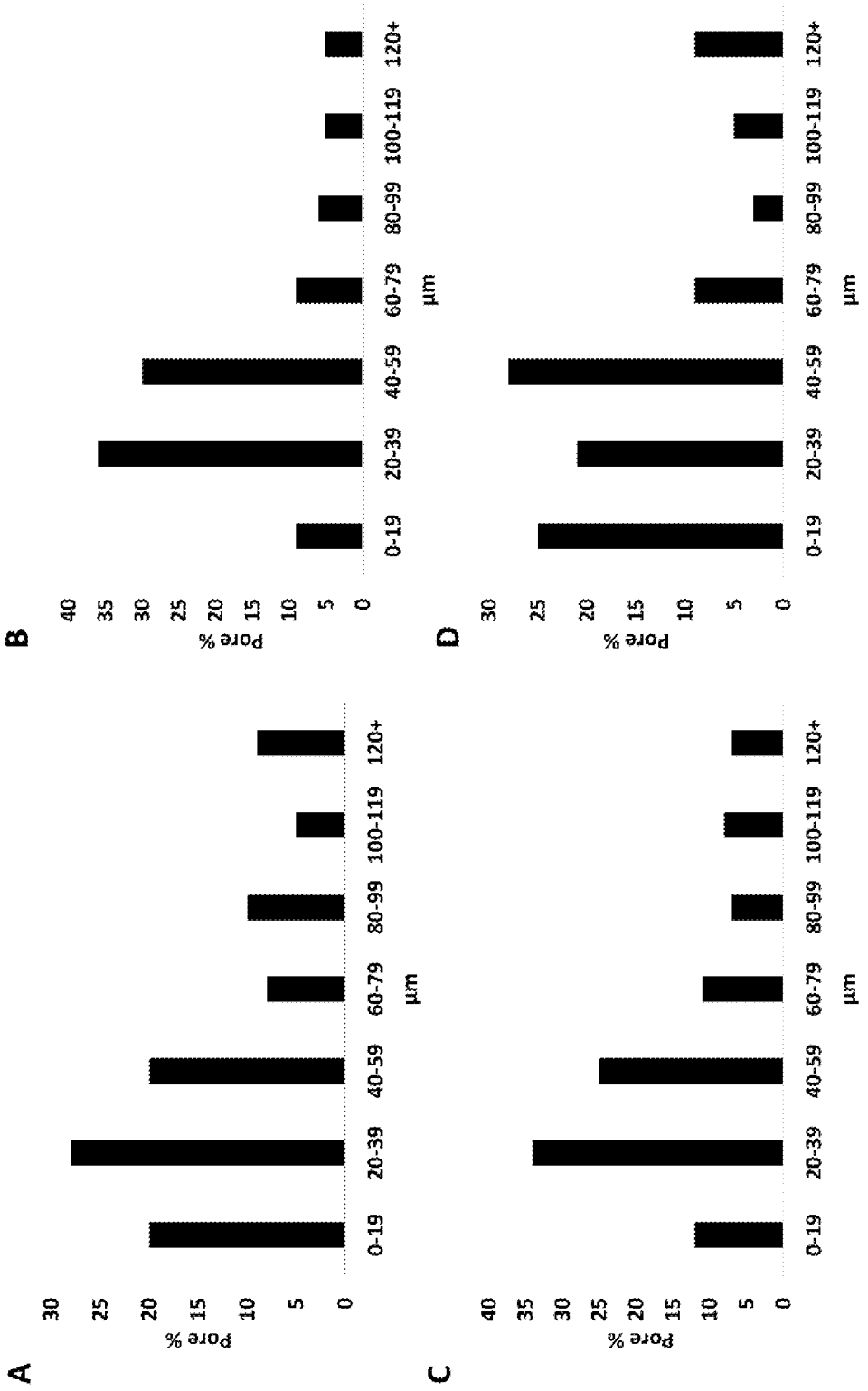


Figure 15

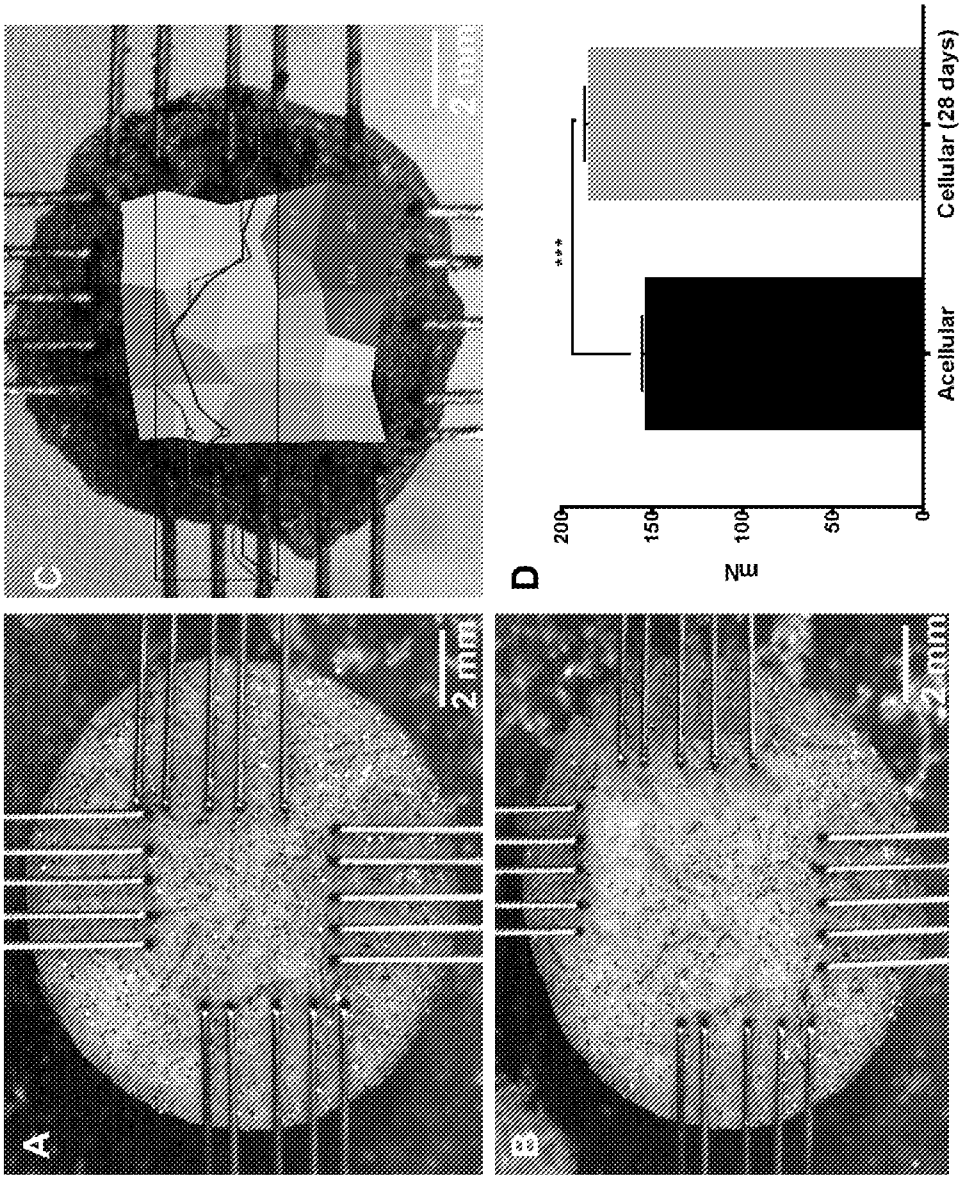

A
2 mm
B
2 mm
C
2 mm
D
***
Acellular
Cellular (28 days)
200
150
100
50
0
kN

Figure 16

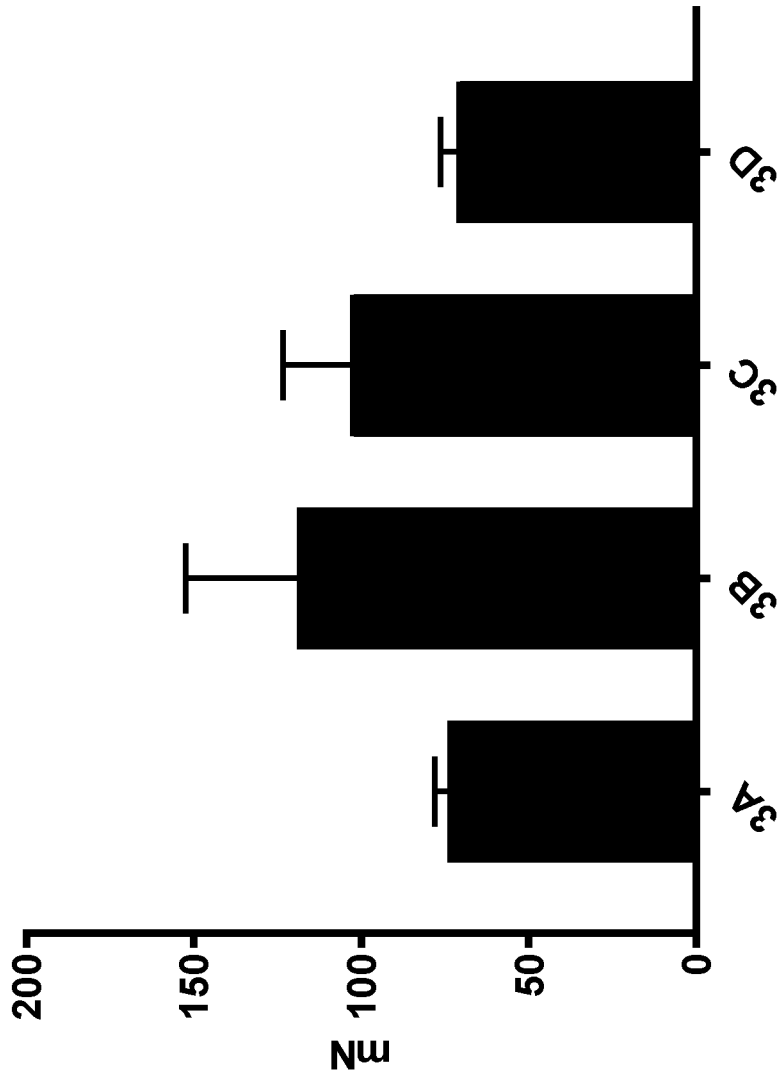


Figure 17

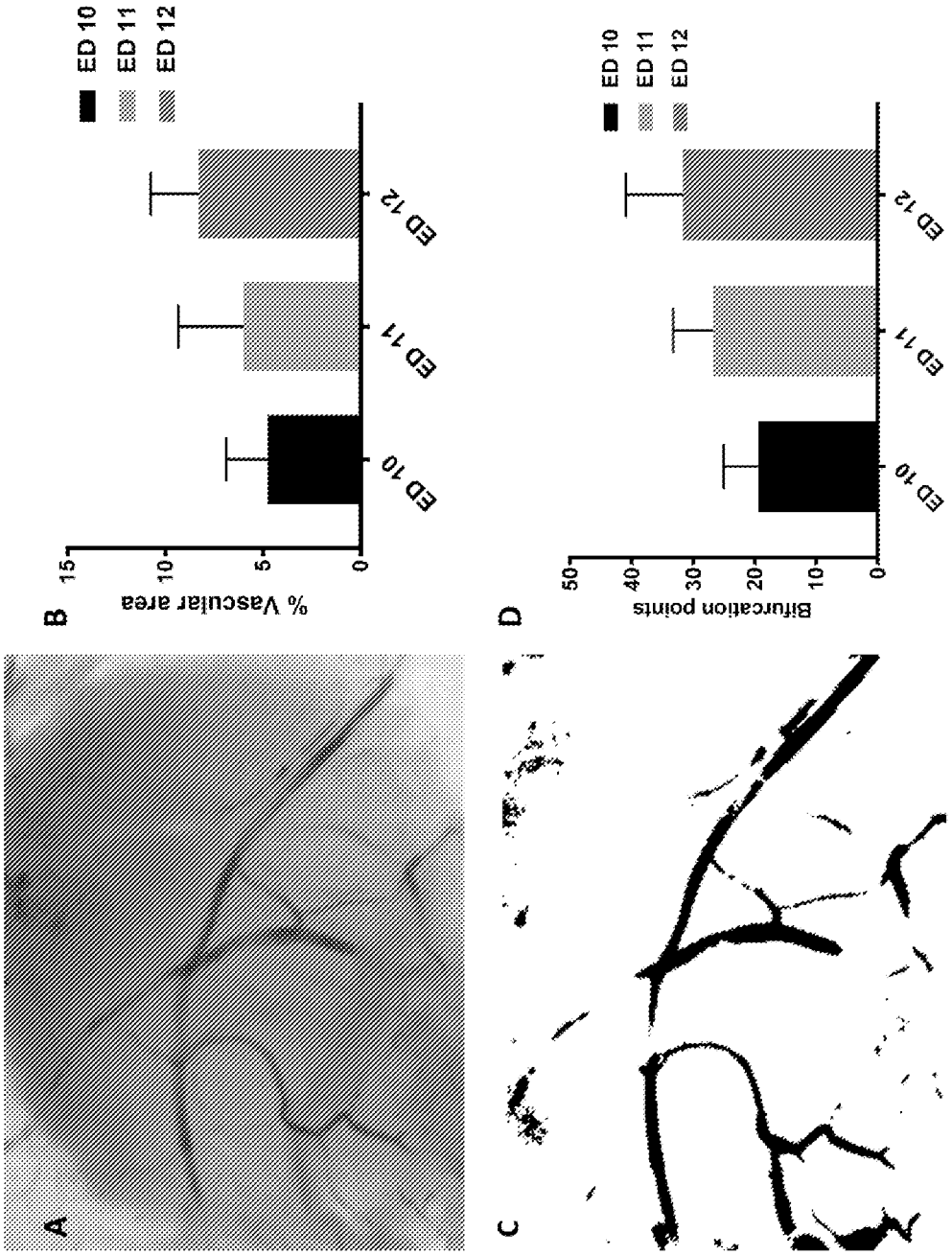

A
B
% Vascular area
15
10
5
0
ED 10
ED 11
ED 12
C
D
Bifurcation points
50
40
30
20
10
0
ED 10
ED 11
ED 12

Figure 18

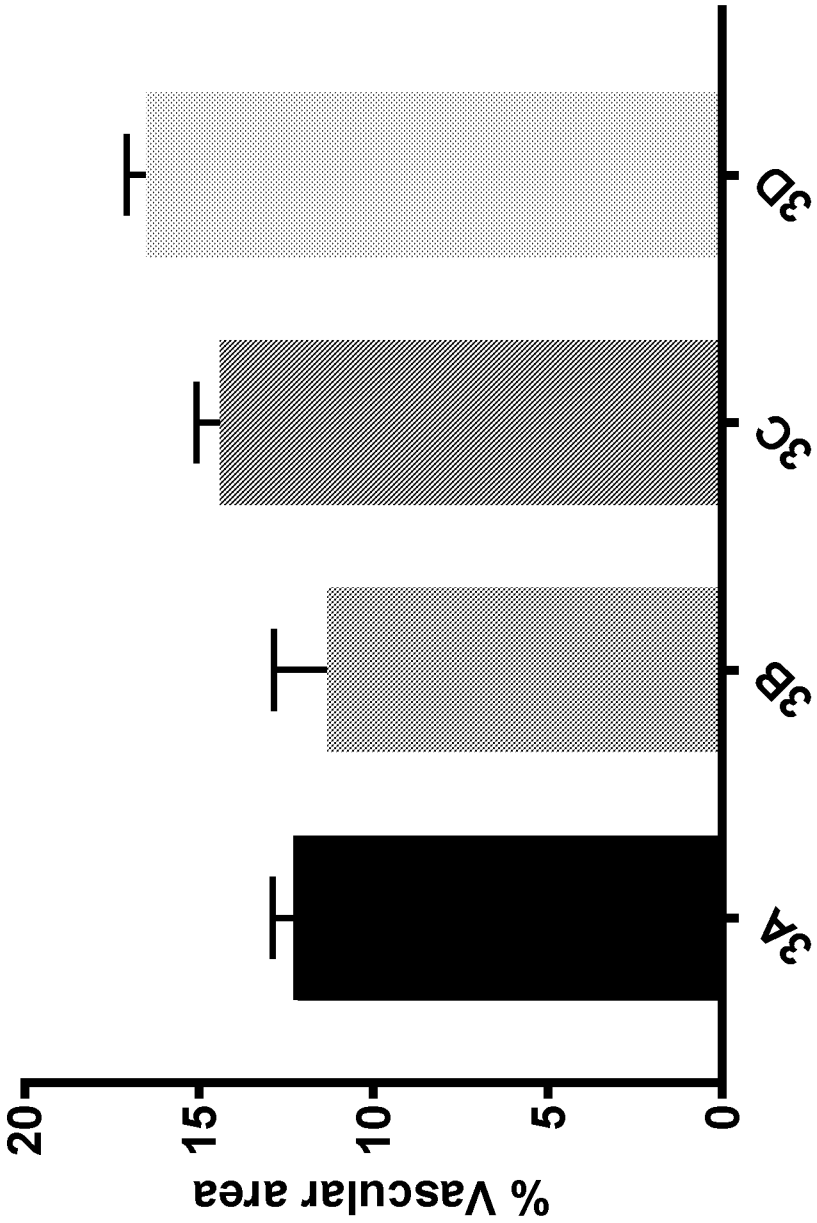


Figure 19

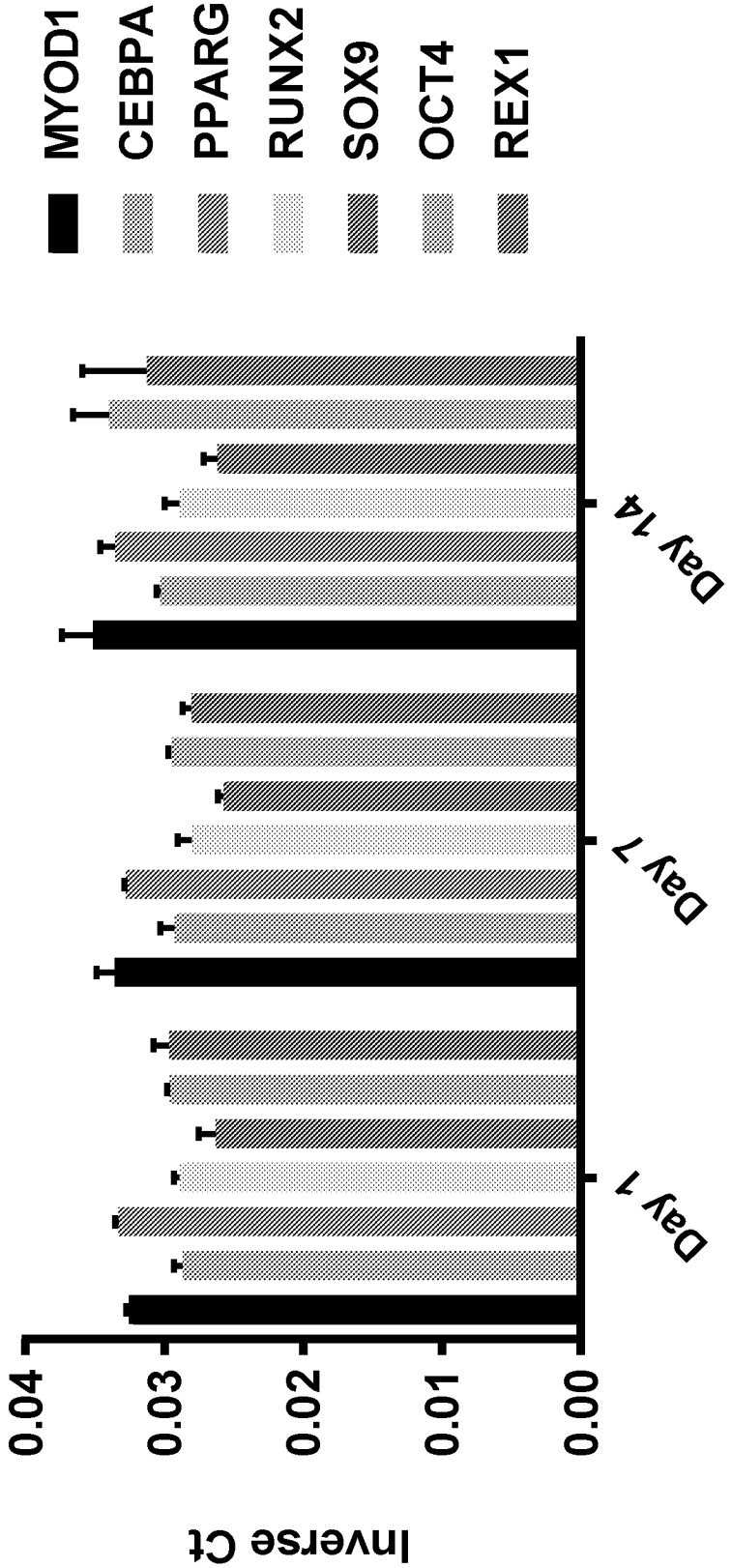

MYOD1
CEBPA
PPARG
RUNX2
SOX9
OCT4
REX1
Inverse Ct
0.04
0.03
0.02
0.01
0.00
Day 1
Day 7
Day 14

Figure 20

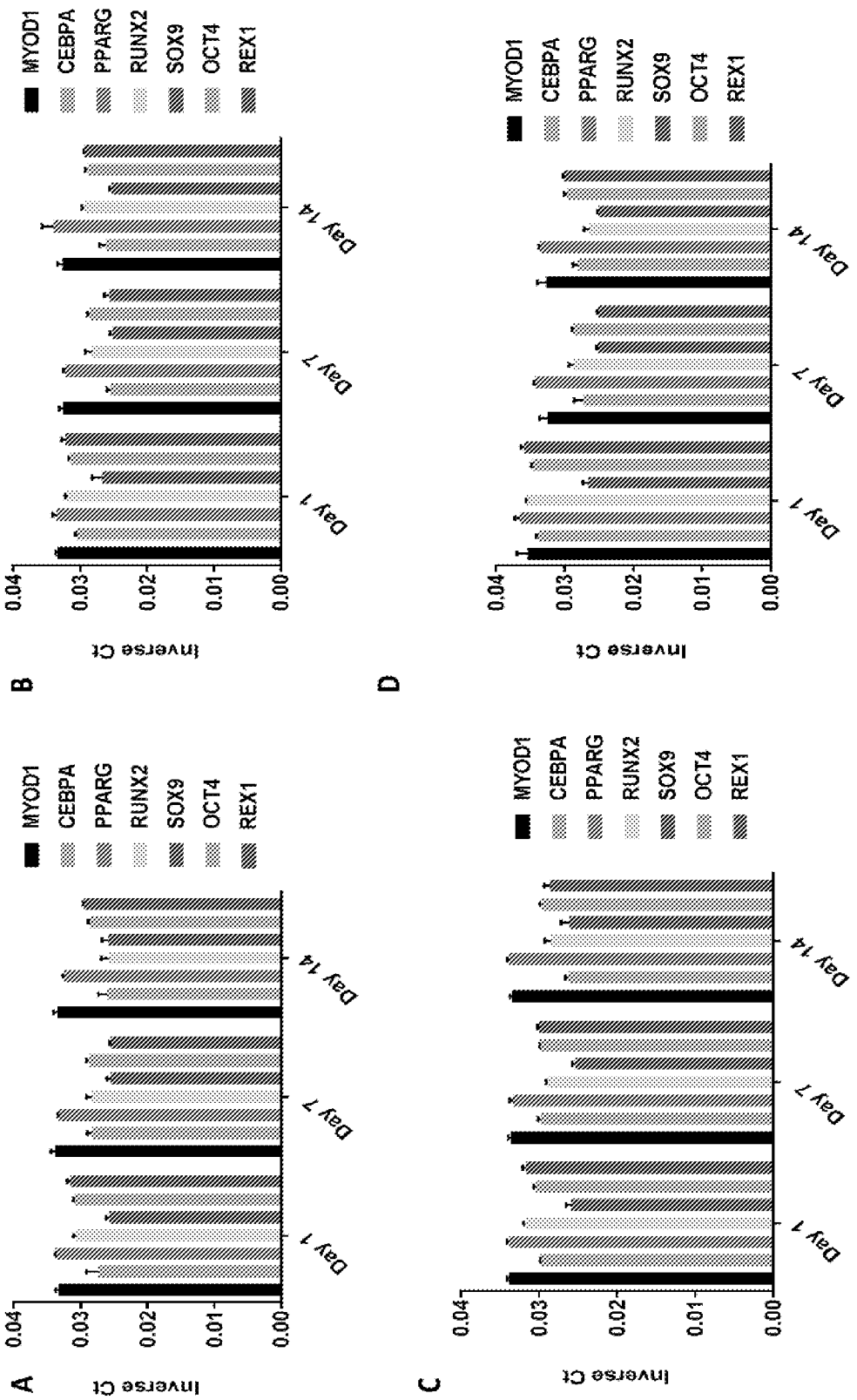

A
B
C
D
MYOD1
CEBPA
PPARG
RUNX2
SOX9
OCT4
REX1
Inverse Ct
0.04
0.03
0.02
0.01
0.00
Day 1
Day 7
Day 14

Figure 21

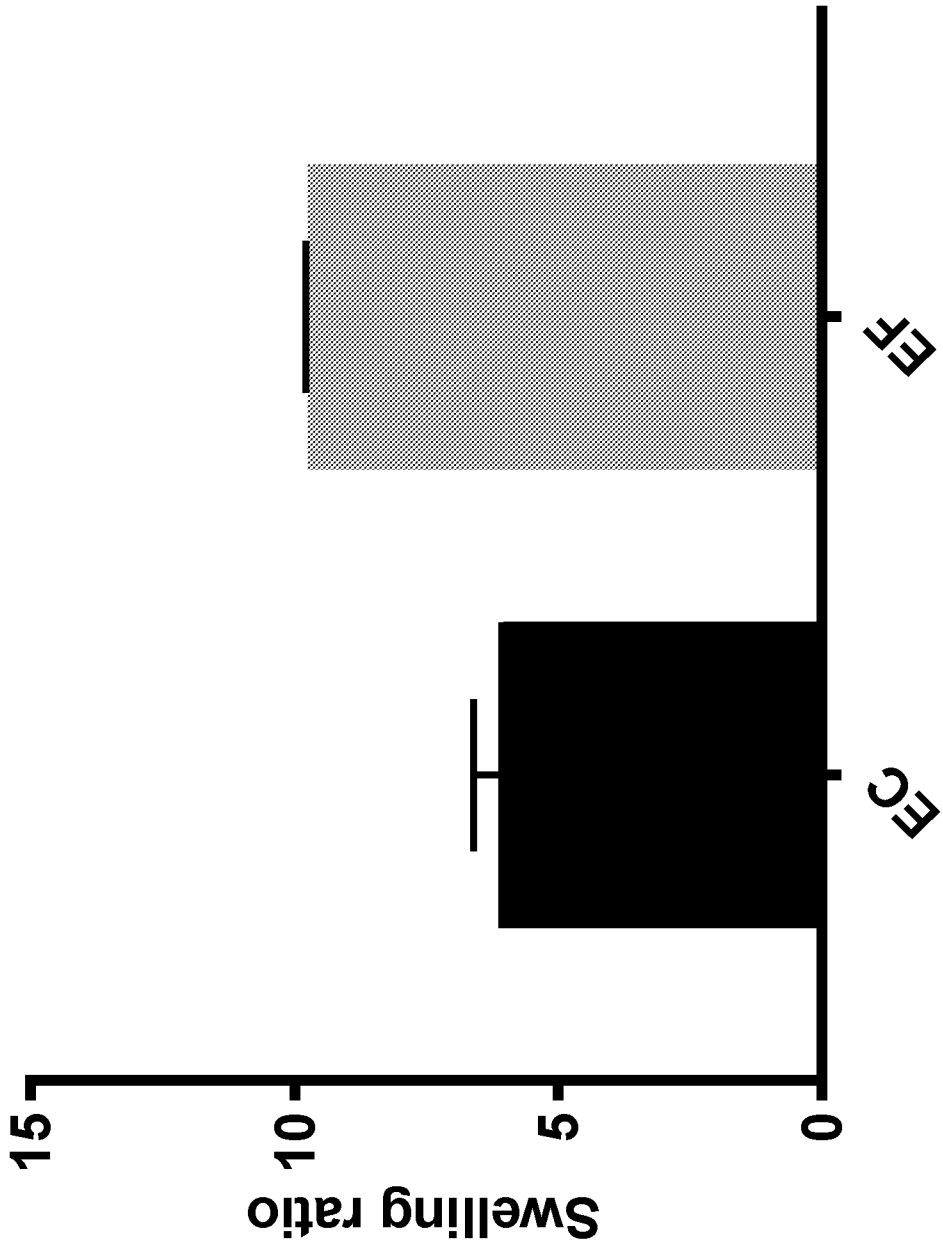


Figure 22

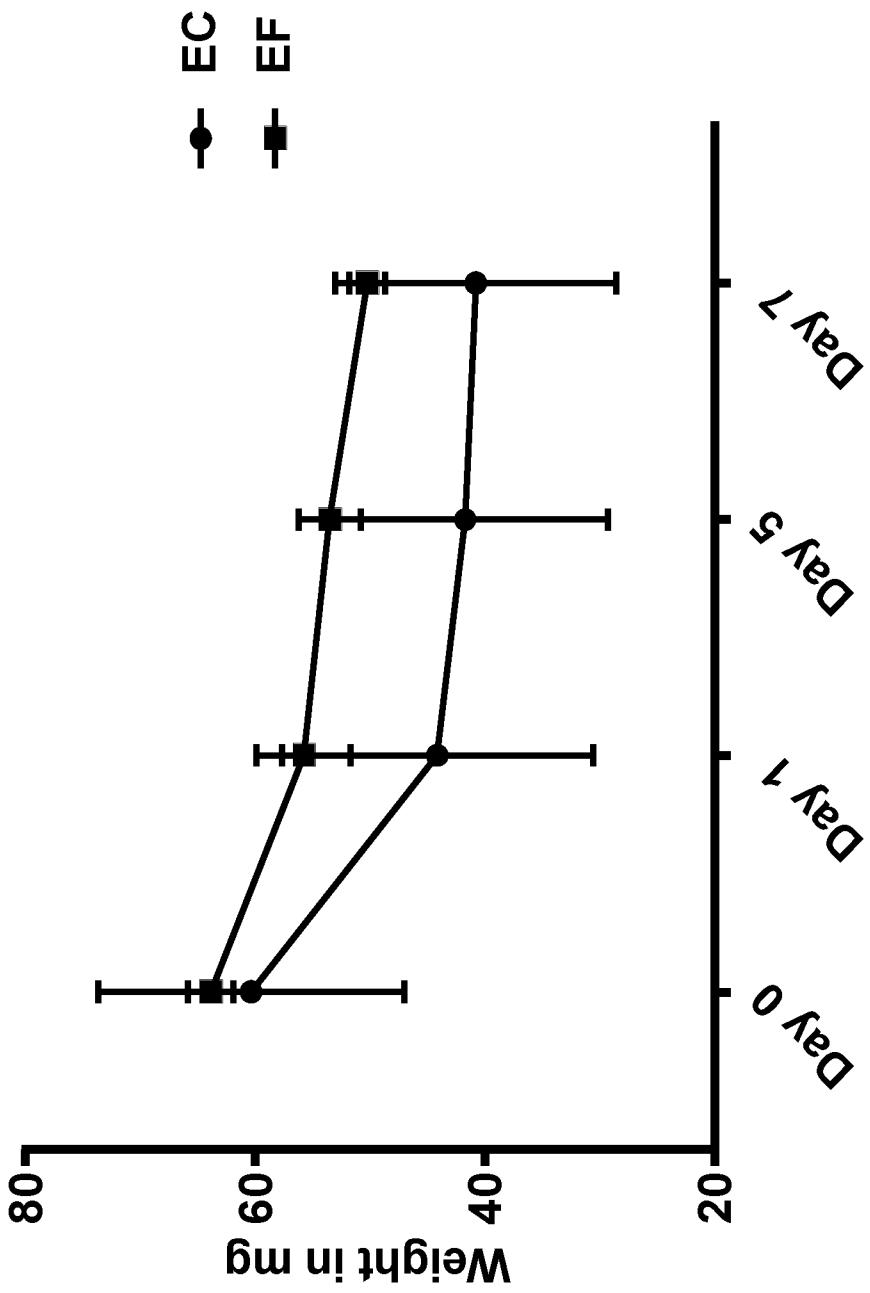


Figure 23

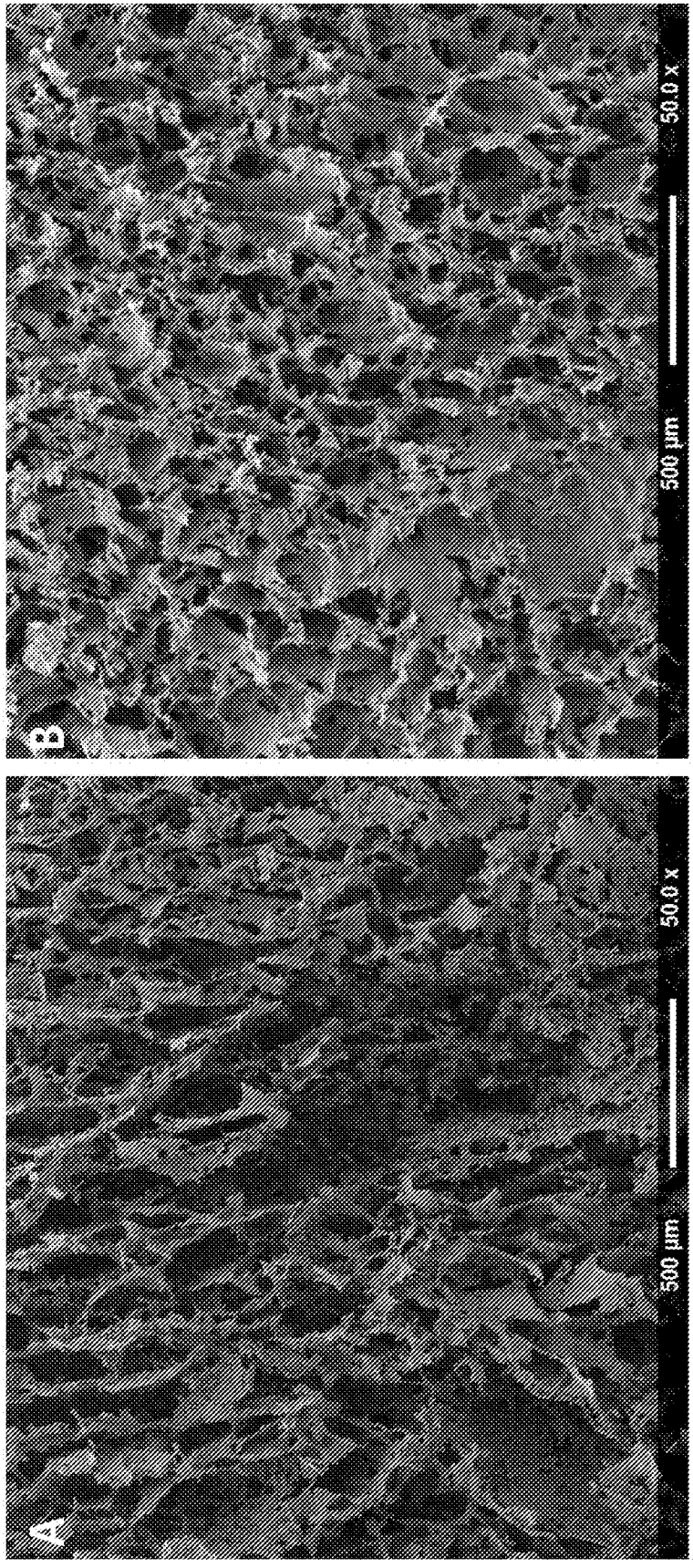


Figure 24

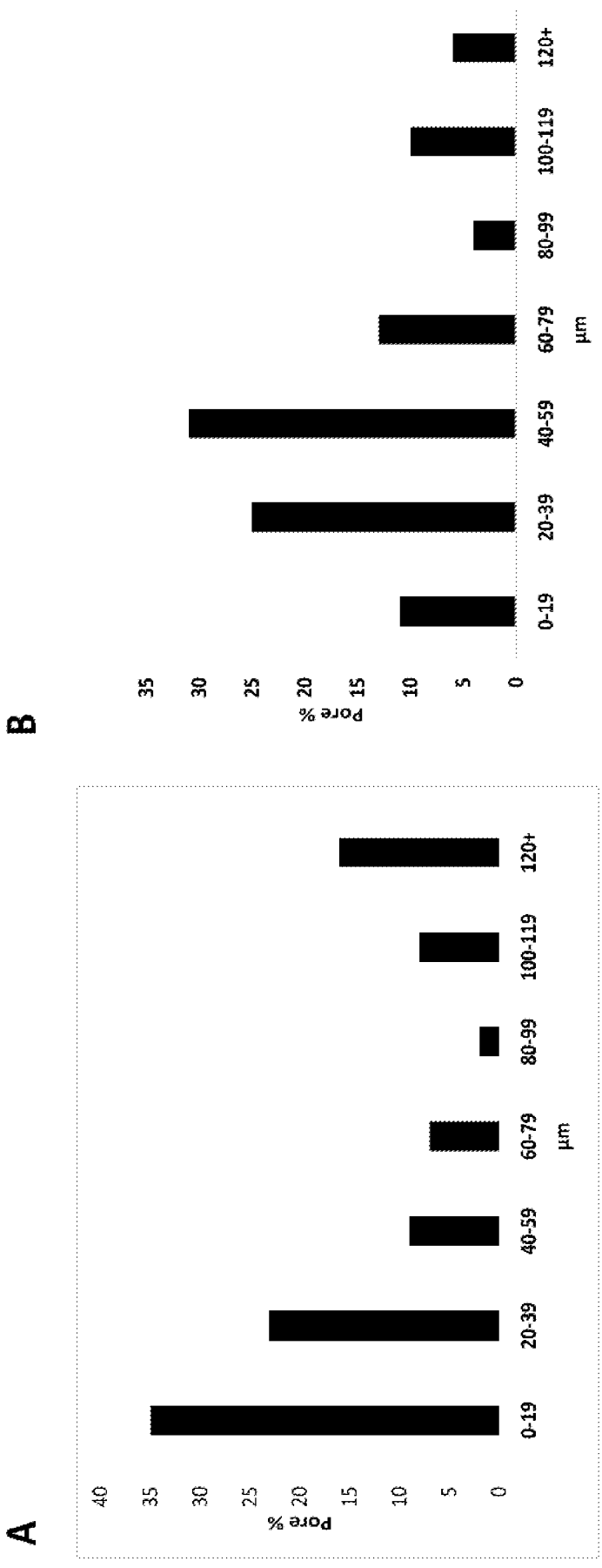

A
Pore %
40
35
30
25
20
15
10
5
0
0-19
20-39
40-59
60-79
80-99
100-119
120+
µm
B
Pore %
35
30
25
20
15
10
5
0
0-19
20-39
40-59
60-79
80-99
100-119
120+
µm

Figure 25

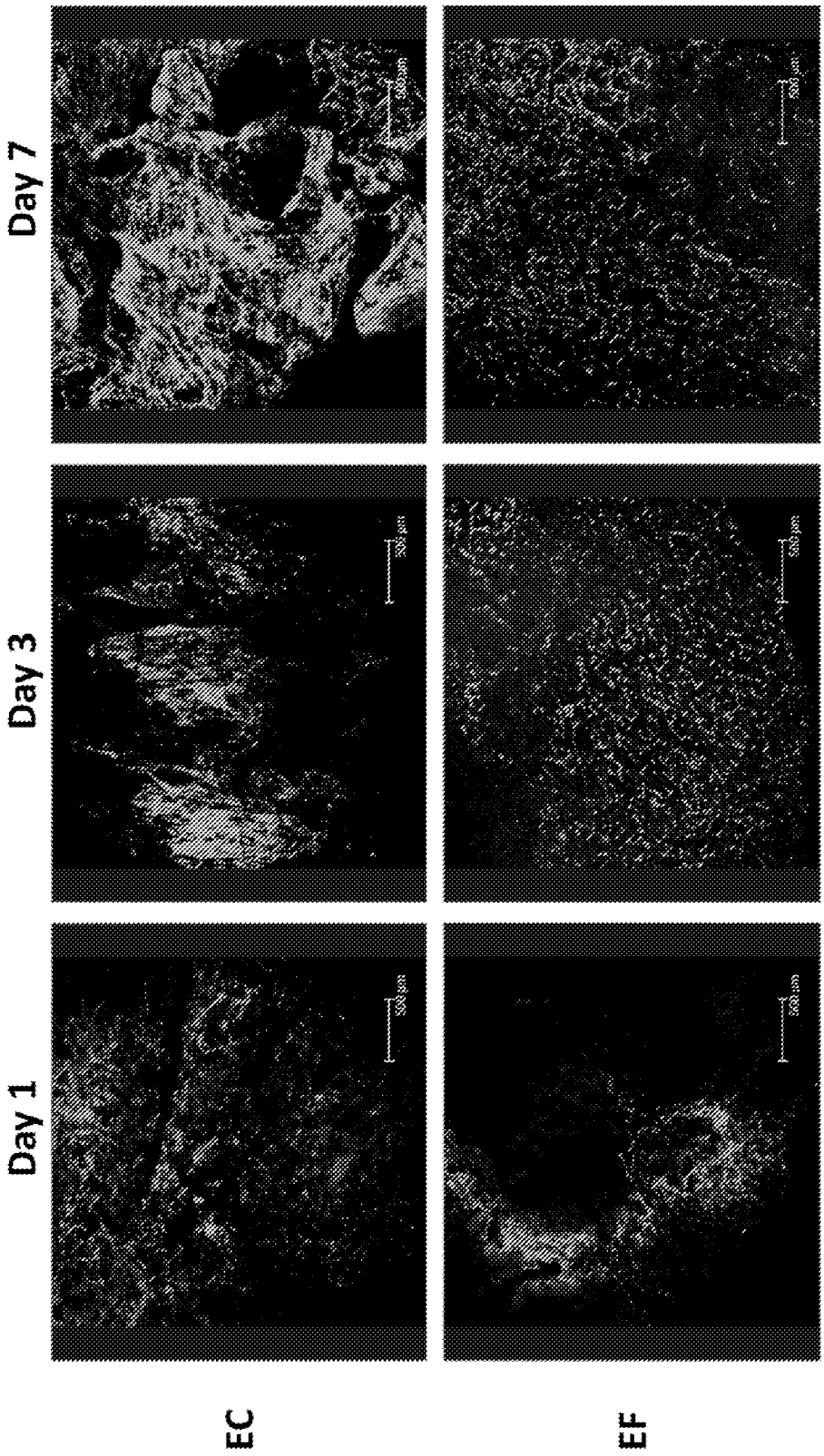


Figure 26

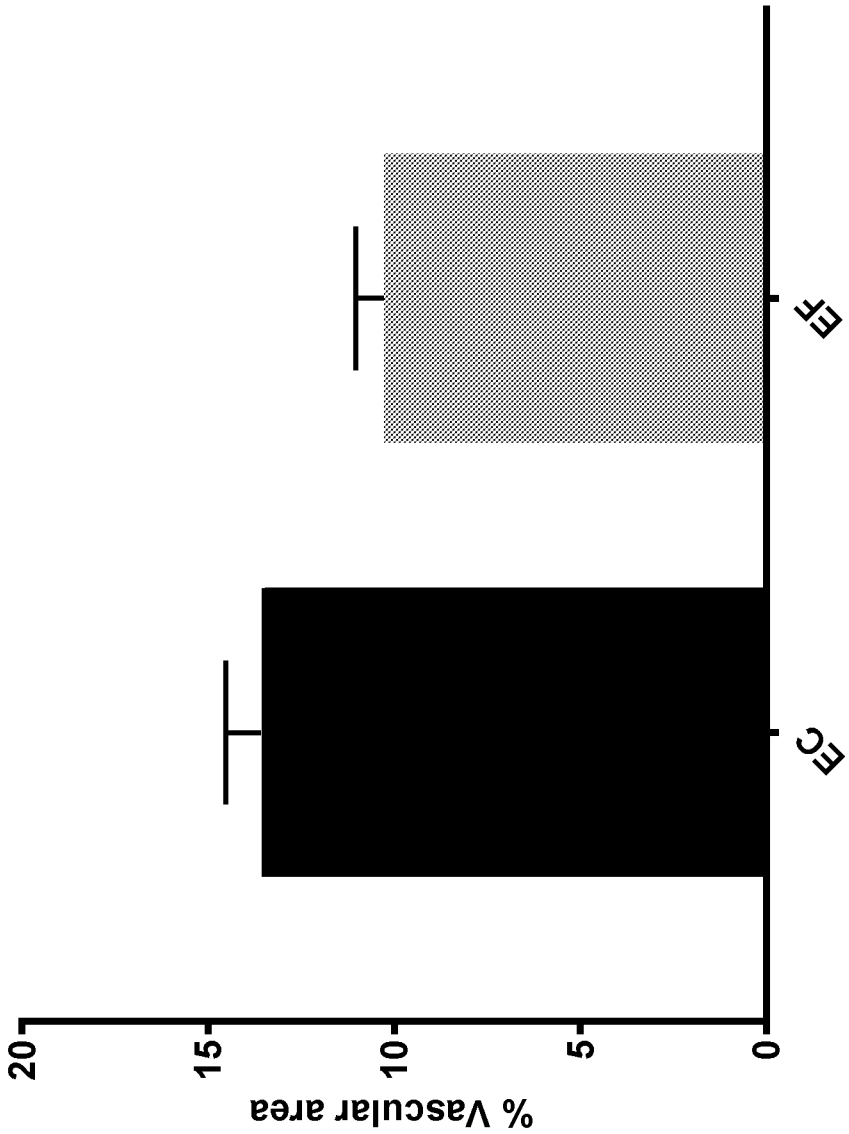


Figure 27

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/052002

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61L27/22 A61L27/36
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61L

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WISE STEVEN G ET AL: "Engineered tropoelastin and elastin-based biomaterials", ADVANCES IN PROTEIN CHEMISTRY AND STRUCTURAL BIOLOGY,, vol. 78, 1 January 2009 (2009-01-01), pages 1-24, XP009193612, DOI: 10.1016/S1876-1623(08)78001-5	1,3,33, 34, 36-38, 46,47
Y	abstract	1-74

X	US 2011/229574 A1 (GUILLEN KARINA H [US] ET AL) 22 September 2011 (2011-09-22)	1,3,33, 34,37, 38,46,47
Y	claim 22; example 2	1-74

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 October 2018	12/10/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Sierra Gonzalez, M

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/052002

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/037028 A1 (NG MARTIN KEAN CHONG [AU]; WEISS ANTHONY STEVEN [AU]; WISE STEVEN GARR) 3 April 2008 (2008-04-03)	1,3,33, 34,37, 38,46,47
Y	claims 1, 12 claim 1	1-74

X	US 2016/143726 A1 (KEMNITZER JOHN E [US] ET AL) 26 May 2016 (2016-05-26)	1,3,33, 34,37, 38,46,47
Y	claim 1	1-74

X	WO 99/45941 A1 (MRS LLC [US]; SANDBERG LAWRENCE B [US]; ROOS PHILIP J [US]; MITTS THOM) 16 September 1999 (1999-09-16)	1,3,33, 34,37, 38,46,47
Y	page 18, lines 7-10; claims 1, 2	1-74

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2018/052002

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 3078388 A1	12-10-2016
		ES 2585388 T3	05-10-2016
		HU E030138 T2	28-04-2017
		PL 2550027 T3	30-11-2016
		PT 2550027 T	12-08-2016
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		US 2015196684 A1	16-07-2015
		US 2017021057 A1	26-01-2017
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		AU 765379 B2	18-09-2003
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		HK 1036225 A1	18-04-2002
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