

Claims

1. A composite scaffold material comprising:
one or both of a crosslinked polymer matrix formed from a non-mammalian collagen and a crosslinking agent and a crosslinked polymer matrix formed from a non-mammalian collagen that has undergone self-crosslinking; and
a plurality of calcium phosphate particles distributed within the crosslinked polymer matrix, wherein the composite scaffold material is porous.
2. The composite scaffold material according to Claim 1, wherein the crosslinking agent is a pharmaceutically acceptable crosslinking agent.
3. The composite scaffold material according to Claim 1 or Claim 2, wherein the crosslinking agent is selected from one or more of the group consisting of genipin and compounds comprising two or more crosslinkable functional groups selected from the group consisting of amino, carboxylic acid, ester, aldehyde and epoxide functional groups (e.g. the crosslinking agent is selected from compounds comprising two or more crosslinkable functional groups selected from the group consisting of aldehyde and epoxide functional groups).
4. The composite scaffold material according to any one of the preceding claims, wherein the crosslinking agent is selected from compounds comprising two crosslinkable functional groups, optionally wherein the crosslinking agent is selected from one or more of the group consisting of glutaraldehyde and 1,4-butanediol diglycidyl ether (e.g. the crosslinking agent is 1,4-butanediol diglycidyl ether).
5. The composite scaffold material according to any one of the preceding claims, wherein when the crosslinked polymer matrix is formed from a non-mammalian collagen that has undergone self-crosslinking, the non-mammalian collagen has been crosslinked by a transglutaminase.
6. The composite scaffold material according to any one of the preceding claims, wherein, when present, the crosslinking agent forms from 3 to 15 wt%, such as from 8 to 10 wt% of the crosslinked polymer matrix formed from a non-mammalian collagen and a crosslinking agent.
7. The composite scaffold material according to any one of the preceding claims, wherein the calcium phosphate particles have a diameter of from 10 nm to 20 μm , such as from 50 nm

to 10 μm , such as from 500 nm to 3 μm , optionally wherein the calcium phosphate particles have a diameter of from 0.5 to 20 μm , such as from 1 to 10 μm , such as from 1.5 to 3 μm , such as about 1.6 μm .

8. The composite scaffold material according to any one of the preceding claims, wherein the calcium phosphate particles are hydroxyapatite, optionally wherein the hydroxyapatite is monophase hydroxyapatite.

9. The composite scaffold material according to Claim 8, wherein the hydroxyapatite is derived from fish scales, optionally wherein the fish scales are from snakehead fish and/or elasmoid scales derived from teleosts.

10. The composite scaffold material according to any one of the preceding claims, wherein the non-mammalian collagen is type I collagen.

11. The composite scaffold material according to any one of the preceding claims, wherein the non-mammalian collagen is derived from bullfrog skin, optionally wherein the bullfrogs belong to the genus *rana* (e.g. the bullfrogs are of the species *rana catesbeiana*).

12. The composite scaffold material according to any one of the preceding Claims, wherein the composite scaffold material has a porosity of from 90 to 99%, such as from 93 to 98.5%, such as 95 to 98%.

13. The composite scaffold material according to any one of the preceding claims, wherein one or more of the following apply:

(ai) the composite scaffold material has a compression modulus of from 0.5 to 4.5 kPa, such as from 1.0 to 4.2 kPa, such as from 1.9 to 4 kPa, such as from 2.5 to 4 kPa, such as from 3 to 3.5 kPa;

(aii) the composite scaffold material is further coated with calcium phosphate particles, optionally wherein the calcium phosphate is hydroxyapatite (e.g. the hydroxyapatite is monophase hydroxyapatite); and

(aiii) more than 50% of the composite scaffold material has degraded after 8 days when subjected to a 1 $\times$ phosphate-buffered saline, and the degradation is measured by bicinchoninic acid (BCA) protein assay kit.

14. Use of a composite scaffold material according to any one of Claims 1 to 13 in the manufacture of a medicament for use in tissue engineering in a subject in need thereof.

15. A composite scaffold material according to any one of Claims 1 to 13 for use in tissue engineering in a subject in need thereof.

16. A method of tissue engineering comprising the step of providing a suitable amount of a composite scaffold material according to any one of Claims 1 to 13 to a subject in need thereof.

17. A method of *in vitro* tissue engineering, wherein the method comprises the steps of:
(bi) supplying a composite scaffold material according to any one of Claims 1 to 13;
(bii) adding cells and a suitable cellular nutrient mixture to the composite scaffold material;
and
(biii) allowing the cells to grow on the composite scaffold material for a period of time.

18. The use according to Claim 14, the composite scaffold material for use according to Claim 15, the method according to Claim 16 and the method according to Claim 17, wherein the tissue engineering is bone tissue engineering.

19. A method of providing a collagen precursor mixture from a non-mammalian source, the method comprising the steps of:

(a) providing a mixture of pre-treated skins from a non-mammalian animal in an acidic solvent; and
(b) subjecting the mixture to mechanical blending to provide the collagen precursor mixture in the form of a paste.

20. The method according to Claim 19, wherein one or more of the following apply:

(ci) the acidic solvent is aqueous acetic acid, optionally wherein the aqueous acetic acid has a molarity of from 0.1 to 1 M, such as from 0.3 to 0.7 M, such as about 0.5 M;
(cii) the acidic solvent is provided in a weight to volume ratio of from 0.1:10 to 2:10, such as 1:10, where the weight refers to the weight of the pre-treated skins from a non-mammalian animal and the volume refers to the volume of the acidic solvent;
(ciii) the blending is conducted over a period of from 1 to 20 minutes, such as from 2 to 10 minutes, such as about 5 minutes; and
(civ) the blending is conducted at from 20,000 to 50,000 rpm, such as from 30,000 to 40,000 rpm, such as about 35,000 rpm;
(cv) the entire method is conducted at a temperature of from 0.1 to 10°C, such as from 1 to 5°C, such as about 4°C;

(cvi) the pre-treated skins are bullfrog skin, optionally wherein the bullfrogs belong to the genus *rana* (e.g. the bullfrogs are of the species *rana catesbeiana*).

21. A method of providing collagen from a non-mammalian source, the method comprising the steps of:

- (aa) providing a collagen precursor mixture in the form of a paste;
- (ab) diluting the paste with water and centrifuging the resulting diluted paste to provide a collagen solution and a pellet comprising pigments and collecting the collagen solution;
- (ac) adding an inorganic salt to the collagen solution for a period of time (e.g. from 12 to 18 hours) to precipitate out a collagen salt, which is then collected by centrifugation;
- (ad) adding an acidic solvent to the collected collagen salt to provide a free collagen mixture and subjecting the free collagen mixture to dialysis to provide a solution of collagen from a non-mammalian source.

22. The method according to Claim 21, wherein one or more of the following apply:

- (ba) the collagen precursor mixture is obtained using the method according to Claim 19;
- (bb) the paste is diluted by water in a ratio of from 1:10 to 1:30 vol/vol, such as from 1:10 to 1:20 vol/vol, such as about 1:10 vol/vol;
- (bc) the centrifugation in step (ab) of Claim 21 is conducted at from 15,000 to 50,000 $\times g$, such as from 20,000 to 35,000 $\times g$, such as about 25,000 $\times g$;
- (bd) the centrifugation in step (ab) of Claim 21 is conducted for a period of from 5 to 45 minutes, such as from 10 to 30 minutes, such as about 15 minutes;
- (be) the inorganic salt in step (ac) of Claim 21 is selected from one or more of sodium sulphate, ammonium sulphate, potassium chloride, and sodium chloride (e.g. the inorganic salt is sodium chloride), optionally wherein the inorganic salt is provided as an aqueous solution having a concentration of from 0.5 to 4.0 M, such as from 0.5 to 1.5 M, such as about 0.9 M;
- (bf) the centrifugation in step (ac) of Claim 21 is conducted at from 3,000 to 10,000 $\times g$, such as from 4,000 to 6,000 $\times g$, such as about 5,500 $\times g$;
- (bg) the centrifugation in step (ac) of Claim 21 is conducted for a period of from 5 to 45 minutes, such as from 10 to 30 minutes, such as about 15 minutes;
- (bh) the acidic solvent in step (ad) of Claim 21 is aqueous acetic acid, optionally wherein the aqueous acetic acid has a molarity of from 0.1 to 1 M, such as from 0.3 to 0.7 M, such as about 0.5 M;
- (bi) the dialysis in step (ad) of Claim 21 is conducted in two rounds, wherein:
 - (iii) the first round dialysis makes use of aqueous acetic acid at a concentration of from 0.01 to 0.3 M, such as from 0.05 to 0.2 M, such as about 0.1 M; and

- (ii) the second round dialysis makes use of water;
- (bj) the entire method is conducted at a temperature of from 0.1 to 10°C, such as from 1 to 5°C, such as about 4°C;
- (bk) step (ac) of Claim 21 is only conducted once.

23. The method according to Claim 21 or Claim 22, wherein the solution of collagen from a non-mammalian source is lyophilised.

24. A method of providing a composite scaffold material according to any one of Claims 1 to 13, wherein the method comprises the steps of:

- (di) providing a solution of a non-mammalian collagen;
- (dii) adding calcium phosphate particles and one or both of a crosslinking agent and an agent to promote self-crosslinking to the solution of a non-mammalian collagen to form a reaction mixture and allowing the reaction mixture to react for a period of time; and
- (diii) removing the solvent to provide the composite scaffold material.

25. The method according to Claim 24, wherein:

after the period of time, the reaction mixture of step (dii) is deposited onto a flat substrate and allowed to dry to provide the composite scaffold material in the form of a film; or

after the period of time, the reaction mixture of step (dii) is deposited into a mold and lyophilised to provide the composite scaffold material in the form of a three-dimensional structure.

26. The method according to Claim 24 or Claim 25, wherein one or more of the following apply:

- (ca) the non-mammalian collagen in the solution of a non-mammalian collagen is provided at a concentration of from 5 to 20 mg/mL, such as about 10 mg/mL;
- (cb) the solvent in the solution of a non-mammalian collagen is aqueous acetic acid, optionally wherein the aqueous acetic acid has a molarity of from 0.1 to 1 M, such as from 0.3 to 0.7 M, such as about 0.5 M;
- (cc) the calcium phosphate particles are provided in a weight to weight ratio relative to collagen of from 0.5:1 to 2:1, such as about 1:1;
- (cd) the crosslinking agent, when present, is provided in an amount of from 3 to 15 wt%, such as from 8 to 10 wt% relative to the dry weight of the collagen;
- (ce) the period of time is from 12 to 48 hours, such as from 18 to 32 hours, such as about 24 hours.