PROCESS FOR PREPARING POROUS COLLAGEN MATRIX FROM CONNECTIVE TISSUE

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The subject invention provides a process for preparing a porous collagen matrix from connective tissue, which comprises treating connective tissue with a solution of hydrogen peroxide and/or an acidic solution so as to obtain a porous collagen matrix.
PROCESS FOR PREPARING POROUS COLLAGEN MATRIX FROM CONNECTIVE TISSUE

FIELD OF THE INVENTION

[0001] The subject invention relates to a process for preparing a porous collagen matrix from connective tissue and a porous collagen matrix prepared by said process.

BACKGROUND OF THE INVENTION

[0002] Collagen is a biodegradable protein and exists in a form of fibers in connective tissue of most animals. The primary function of collagen is to maintain the integrity of tissues and to provide tensile strength essential to tissues. Collagen molecule is a biological macromolecule composed of three polypeptide chains that twist around one another. Each polypeptide is composed of more than one thousand amino acids, wherein the primary amino acids are glycine, proline and hydroxyproline. At present, more than 21 different types of collagen have been discovered.

[0003] For applications, collagen can be manufactured in different forms, such as sponge, gel, tube, sheet, etc. They can be applied as hemostats, wound dressings, drug carriers, scaffolds of artificial organs, fillers to recover tissues, microcarriers and macracarriers for supporting cell growth, etc. In order to make the above referred collagen matrix existing a porous structure to facilitate cell migration, cell growth or encapsulation and release of drugs, collagen is usually isolated from connective tissue and fabricated into a porous matrix through a lyophilization step. Generally, the matrix is treated with a cross-linking agent and is frozen at various temperatures followed by vacuum drying during the lyophilization step.

[0004] The preparation of porous collagen matrix has been disclosed in many prior art patents. For instance, in U.S. Pat. No. 4,193,813, the concentrated collagen at pH 3.5 to pH 6.5 is crosslinked with glutaraldehyde followed by freezing at 0 to -20°C. After thawing, the water of the frozen material is eliminated to form a sponge matrix. The pore size of the matrix formed by this process is about 80-1400 μm.

[0005] U.S. Pat. No. 4,412,947 relates to a process that pure insoluble particulate collagen is suspended in a weak aqueous organic acid solution followed by freeze-drying at -60 to -70°C with a temperature reduction rate of -0.3 to -0.4°C per minute, and then lyophilized to form a porous collagen sheet. U.S. Pat. No. 4,522,753 relates to a process of mixing collagen and chondroitin sulfate to form a copolymer material. The material is then cross-linked by glutaraldehyde and lyophilized to form a porous matrix with a pore size of 20-180 μm. Such matrix can be used as a basic material of synthetic skin grafts.

[0006] U.S. Pat. No. 4,970,298 discloses a collagen matrix prepared by dispersing collagen in an acidic solution or by mixing the collagen dispersion with hyaluronic acid and fibronectin. The dispersion is frozen at the different temperatures and then lyophilized to form a porous sponge. The sponge is cross-linked with a carbodimide or by a dehydrothermal process. The freezing temperature is -30°C to -50°C. The pore size of the matrix obtained is about 20-250 μm. The collagen matrix containing hyaluronic acid or fibronectin exhibits a pore size of 100-150 μm.

[0007] U.S. Pat. No. 4,948,540 describes a process that involves freeze-drying the mixture of native collagen and soluble collagen fibers and compressing at a pressure of 15,000-30,000 p.s.i. The material is then cross-linked by dehydrothermal method to obtain a final product which is a sheet material with high absorptivity.

[0008] U.S. Pat. No. 5,116,552 describes a process for preparing a crack-free sponge matrix. An acidic collagen solution is frozen at -40°C and lyophilized into a sponge. The sponge is then incubated at 105°C for 24 hours and then cross-linked for 24 hours with glutaraldehyde to form a matrix with a pore size of 50-120 μm. The matrix is then immersed in 15% alcohol. After second lyophilization at a lower temperature of -80°C or -135°C, a crack-free sponge matrix is obtained.

[0009] U.S. Pat. No. 5,689,080 describes a process for preparing an absorbable implant material. A sponge matrix is formed by adding a proper amount of alcohol to the collagen dispersion in sodium hydroxide, pre-freezing it at a low temperature (about -5°C), adding ice particles to the dispersion, cross-linking the dispersion with hexamethylene disocyanate (HMDI), and followed by lyophilizing the dispersion. The matrix obtained by this process exhibits a pore size of 50-400 μm.

[0010] The processes for the preparation of the collagen matrix disclosed in the aforementioned patents comprise complicated operation steps which include extraction and purification of collagen. Acidic or alkaline collagens are generally used and cross-linked by a dehydrothermal process or by the chemical cross-linking agents, and lyophilized to obtain porous collagen matrices. The matrix products obtained by any of these processes exhibit poor porous homogeneity. Furthermore, because most of the chemical cross-linking agents are toxic, the application extent of these processes is limited.

[0011] To obtain a better porous homogeneity, to prevent possible toxic effects resulted from the chemical cross-linking agents, to save vast energy on collagen extraction, and yet to obtain a more stable porous network, the subject invention proposes an improved process for manufacturing the porous collagen matrix for collagen-related products and applications.

SUMMARY OF THE INVENTION

[0012] The subject invention provides an improved method and products thereof that overcome the disadvantages of the conventional technique for preparing a collagen matrix. The operation steps of the process of the subject invention are simple and do not need to use a cross-linking agent. Meanwhile, the subject invention provides a collagen matrix product with excellent qualities with unexpected results.

[0013] An object of the subject invention is to provide a process for preparing a porous collagen matrix from connective tissue.

[0014] Another object of the invention is to provide a porous collagen matrix prepared by the process of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Additional objects and features of the present invention will become more apparent and the invention itself
will be best understood from the following Detailed Description of the Invention, when read with reference to the accompanying drawings.

[0016] FIG. 1 shows the cross section of the porous collagen matrix prepared by one embodiment of the present invention illustrated in the process of Example 1.

[0017] FIG. 2 shows the cross section of the porous collagen matrix prepared by another embodiment of the present invention illustrated in the process of Example 2.

[0018] FIG. 3 shows the cross section of the porous collagen matrix prepared by still another embodiment of the present invention illustrated in the process of Example 3.

[0019] FIG. 4 shows the cross section of the porous collagen matrix prepared by one further embodiment of the present invention illustrated in the process of Example 4.

[0020] FIG. 5 shows the cross section of the porous collagen matrix prepared by one alternate embodiment of the present invention illustrated in the process of Example 5.

DETAILED DESCRIPTION OF THE INVENTION

[0021] The preferred embodiments of the present invention described below relate particularly to a porous collagen matrix and innovative processes for making same. While the description sets forth various embodiment specific details, it will be appreciated that the description is illustrative only and should not be construed in any way as limiting the invention. Furthermore, various applications of the invention, and modifications thereto, which may occur to those who are skilled in the art, are also encompassed by the general concepts described below.

[0022] The process of the subject invention overcomes the disadvantages of the conventional technique of preparing collagen matrices. The process of the subject invention has the following features and advantages: (i) the cost of manufacturing the porous collagen matrix is substantially reduced; (ii) the preparation time is greatly decreased; (iii) the structure of the matrix is more stable than the conventionally prepared collagen matrix; (iv) without the use of cross-linking agents; and (v) the collagen matrix prepared by the process of the subject invention can be readily used directly as biomedical materials or scaffolds for tissue engineering.

[0023] The subject invention utilizes animal connective tissue as starting materials. Then the material is subject to the necessary treatment procedures (such as the treatment with a chemical solution and/or bactericide, and low-temperature freezing and lyophilization under vacuum) to form porous structure.

[0024] Therefore, it is some aspects of the subject invention to provide a process for the preparation of a porous collagen matrix. Said process comprises treating connective tissue with a solution of hydrogen peroxide so as to obtain a porous collagen matrix.

[0025] It is another aspect of the subject invention to provide a process for preparing a porous collagen matrix from connective tissue, wherein the process comprises treating connective tissue with an acidic solution so as to obtain a porous collagen matrix.

[0026] It is still another aspect of the subject invention to provide a process for preparing a porous collagen matrix from connective tissue, wherein the process comprises treating connective tissue with an acidic solution followed by a hydrogen peroxide solution, freezing said connective tissue at a proper temperature under a proper temperature reduction rate, and lyophilizing said connective tissue so as to obtain a porous collagen matrix.

[0027] Collagen is rich in animal connective tissue. Therefore, the subject invention may directly utilize connective tissue as the starting materials. The source of connective tissue may derive from animals which have connective tissue, such as cattle, pigs, horses, sheep, chickens, ducks, turkeys, geese, whales, sharks, and the like. The connective tissue suitable for the process of the subject invention includes skin, dermis, subcutaneous tissue, ligament, tendon, aponeurose, cartilage, bone tissue, and the like. The following description of dermis treatment is an example of the subject invention. Lipid of fresh animal skin is removed, and then the animal skin is washed a few times with saline. The surface layer of the animal skin is removed with a dermatome, and then the dermis with proper thickness is obtained. The dermis is washed with phosphate buffered saline. Solution retained on the surface of the dermis is generally removed by absorption. The dermis can be subject to the subsequent treatments with chemical reagents.

[0028] In one embodiment, the process of the subject invention utilizes an acid to treat connective tissue so that the connective tissue can be softened. The acids suitable for the subject invention include organic or inorganic acids. Inorganic acids may include, but are not limited to, hydrochloric acid, phosphoric acid, boric acid or sulfuric acid.

[0029] Organic acids may include, but is not limited to, peracetic acid, formic acid, acetic acid, propanoic acid, butyric acid, pantanoic acid (valeric acid), hexanoic acid (caproic acid), heptanoic acid (enanthic acid), capric acid, oxalic acid, malonic acid, succinic acid, glutaric acid, adipic acid, benzoic acid, and the analogs, or derivatives of the aforementioned acids, such as methylpantoanoic acid, 2-hydroxypropanoic acid (lactic acid), chlorohexanoic acid, 4-hydroxy-6-methylheptanoic acid, 2-amino propanoic acid, 2,3-dihydroxy succinic acid, butenedioic acid (fumaric acid or maleic acid), methylbenzoic acid, chlorobenzoic acid, hydroxybenzoic acid, phthalic acid, cyclohexanoic acid, methylocyclobutanecarboxylic acid, methylcyclhexane carboxylic acid, cyclohexaneedicarboxylic acid, etc.

[0030] In the step of acid treatment, the proper concentration of the acid varies with the species of the acid. For instance, the concentration of acetic acid is preferably at about 0.01 to 4M. In that case, dermal tissue is reacted at 4 to 45 for 1 to 150 hours. The concentration of hydrochloric acid is preferably at about 0.01 to 2N. In that case, dermal tissue is reacted at 0 to 60 for 0.1 to 150 hours.

[0031] The acid treatment can be carried out in two steps. For instance, dermal tissue is initially treated with an acidic solution at higher concentration of 0.1 to 6M for 0.1 to 100 hours in the first step, and then with an acidic solution at lower concentration of 0.01 to 2M for 1 to 200 hours in the second step. For instance, one of the preferred embodiment of the subject invention is that connective tissue is treated with 1.0M of acetic acid solution on a rotator at 37 for 12 hours; then the treated connective tissue is placed in 0.2M of
acetic acid solution for 72 hours so as to achieve the effects of penetration and immersion of acids in both of inner and outer parts of the connective tissue.

[0032] In the step of the acid treatment, salts can be added to the acidic solution so as to stabilize collagen molecules to create a better porous structure in the matrix. For instance, the concentration of a salt is preferably at about 0.01 to 4M. Salts suitable for the subject invention include organic or inorganic salts. Inorganic salts may include, but are not limited to, halide salt of alkaline or alkaline earth group, such as sodium chloride or calcium chloride. Organic salts may include, but are not limited to, carboxylates, such as calcium propanoate, sodium benzoate, sodium acetate or sodium carbonate. When 0.1 to 2M of sodium chloride is added to the acidic solution, the best effect for stabilizing the matrix can be achieved, and the best porosity can be obtained. For instance, in the process of the subject invention mentioned above, a collagen matrix with stable and homogeneous pores can be formed if 0.5M of sodium chloride is added to the lower concentration (0.2M) of acetic acid solution.

[0033] The hydrogen peroxide treatment can simultaneously kill bacteria and produce pores in the matrix. The concentration of hydrogen peroxide solution suitable for the process of the subject invention is 0.1% to 10%, preferably 0.5% to 3.0%. This treatment can be carried out at 0 to 70, preferably 4 to 60, for 0.1 to 240 hours. Said treatment can be carried with peracetic acid, NaClO₃, periodic acid, perboric acid, hydroxide or halide containing tertiary amino group, such as d-tubocurarine chloride, choline chloride, muscarine hydroxide, acetylcholine hydroxide, betaine, decamethonium chloride, hexamethonium chloride, etc. In addition, other chemical agents, such as hydriodic acid, trichloroacetic acid, dihydroxyacetic acid, chloride dioxide, polyvinyl pyrrolidone-iodine (povidone-iodine), p-aminosalicylic acid,isonicotinic acid hydrazine, sulfonamides, trimethoprim, metronidazole, 4-quinolone derivatives, imidazole derivatives, azithromycin, etc., can be used in this step to achieve the effect similar to the hydrogen peroxide treatment.

[0034] To facilitate that the porous collagen matrix can be directly utilized as artificial skins or tissue scaffolds, the process of the subject invention may further comprise a step of removing non-collagenous impurities. Said step utilizes a solution containing a detergent, such as sodium dodecyl sulphate (SDS), Tego compounds (such as Tween 80, Triton W. R. 1339, p-isooctylpolyoxy-ethylene phenol polymer; Triton A20), cetylpyridinium chloride, ceteryltrimethyl-ammonium bromide, dioctyl sodium sulphosuccinate, Emasol 4130 (polyoxyethylene sorbitan monoleate), Lubrol W, Nonidet P40, etc. Preferably, a solution containing 0.01 to 10% of SDS can be used to treat the connective tissue at 4 to 45 for 1 to 150 hours.

[0035] The solution containing a detergent used in the process of the subject invention may further comprise a chelating agent, such as EDTA (ethylene diamine tetraacetic acid), DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid), DOTP (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetakis(methylene phosphonic acid)), CDTA (trans-1,2-diaminocyclohexanetetra-acetic acid), Tiron (4,5-dihydroxybenzene-1,3-disulphonic acid), thiourea, 8-hydroxyquinoline-5-sulphonic acid, 3,6-disulpho-1,8-dihydroxy-naphthalene, Eriochrome black T (1-(1-hydroxy-2-naphthylazo)-2-hydroxy-5-nitro-4-naphthalene sulphonyl acid), ammonium perpuretate, etc. For instance, the chelating agent is preferably EDTA with a concentration of 0.01 to 100 mM. Regarding the aforementioned solutions, when the connective tissue is treated with a solution containing only SDS, a collagen matrix with smaller pore size is obtained.

[0036] After the treatments with the aforementioned chemical agents, the connective tissue can be subject to lyophilization. In the prior freezing step of lyophilization, the temperature reduction rate and the final freezing temperature are associated with the pore size and homogeneity of the matrix. For example, when the collagen matrix is frozen at −20, the homogeneity of pores in the said matrix is more proper for cell ingrowth; when the collagen matrix is frozen at 80, the pore size of the matrix is smaller.

[0037] It is one object of the subject invention to provide the porous collagen matrices prepared by the aforementioned processes. The porous collagen matrix prepared by the processes of the subject invention can be used in the preparation of artificial organs or biomedical materials, as the media for cell culture, or used in tissue engineering.

[0038] The following examples are for further illustration of the invention but not intended to limit the invention. Any modifications and applications by persons skilled in the art in accordance with the teachings of the invention should be within the scope of this invention.

**EXAMPLE**

**[0039] Example 1**

**[0040] Example 2**

**[0041] Example 3**

**[0042] Example 4**
The dermal tissue was then treated with a solution of 0.5N hydrochloric acid on a rotator at 37 for 3 hours. The treated dermal tissue was then washed with an aseptic phosphate buffered saline solution so as to remove residual chemical agents. A porous collagen matrix was obtained and the cross section of the matrix was evaluated and photographed under a light microscope as shown in FIG. 3.

Example 4

Lipid and fat of pigskin were removed and washed twice. Epidermis of pigskin was removed with a dermatome, and dermal tissue with thickness of 0.5 mm was selected. The dermal tissue was washed with a phosphate buffered saline solution containing 0.02% NaCl. Solution retained on the surface of the dermal tissue was removed by absorption.

The dermal tissue was then treated with a solution of 0.5N hydrochloric acid on a rotator at 37 for 6 hours. The dermal tissue was then washed with an aseptic phosphate buffered saline solution to remove residual chemical agents. Solution retained on the surface of the dermal tissue was removed by absorption. The dermal layer was then frozen at −20 for lyophilization. A porous collagen matrix was obtained and the cross section of the matrix was evaluated and photographed under a light microscope as shown in FIG. 4.

Example 5

Lipid and fat of pigskin were removed and washed twice. Epidermis of pigskin was removed with a dermatome, and dermal tissue with thickness of 0.5 mm was selected. The dermal tissue was washed with a phosphate buffered saline solution containing 0.02% NaCl. Solution retained on the surface of the dermal tissue was removed by absorption.

The dermal tissue was then treated with a solution of 1.0M acetic acid on a rotator at 37 for 12 hours. The dermal tissue was then transferred to a solution of 0.2M acetic acid containing 0.5M sodium chloride and reacted for 72 hours. After treatment with acetic acid solution, the dermal tissue was transferred to a solution of 1% hydrogen peroxide for reaction for 24 hours and then to a solution containing SDS and EDTA for reaction at 37 for 24 hours.

After completing the aforementioned treatments with the chemical solutions, the dermal tissue was washed with aseptic phosphate buffered saline solution to remove the residual chemical agents. The dermal tissue was then frozen at −20 for lyophilization. A porous collagen matrix was obtained and the cross section of the matrix was evaluated and photographed under a light microscope. As shown in FIG. 5, the homogeneity of pores and the pore size of the matrix are very suitable for applications.

Although preferred embodiments of the invention have been described in detail, including a porous collagen matrix and methods of manufacturing thereof, certain variations and modifications will be apparent to those skilled in the art, including embodiments that do not provide all of the features and benefits described herein. Accordingly, the scope of the present invention is not to be limited by the illustrations or the foregoing descriptions thereof, but rather solely by reference to the appended claims.

We claim:

1. A process for preparing a porous collagen matrix from connective tissue, said process comprising treating connective tissue with a solution of hydrogen peroxide effective for preparing the porous collagen matrix.

2. The process according to claim 1, wherein the solution of hydrogen peroxide has a concentration between 0.1 and 10%.

3. The process according to claim 1, wherein the connective tissue is treated at a temperature ranging from 4 to 60.

4. The process according to claim 1, the process further comprising steps of freezing the connective tissue treated with a solution of hydrogen peroxide, wherein the step of freezing is carried out at a proper temperature with a proper temperature reduction rate, and lyophilizing the connective tissue effective for preparing the porous collagen matrix.

5. A process for preparing a porous collagen matrix from connective tissue, said process comprising treating connective tissue with an acidic solution effective for preparing the porous collagen matrix.

6. The process according to claim 5, wherein the acidic solution is selected from a group consisting of peracetic acid, hydrochloric acid, oxalic acid, acetic acid and sulfuric acid.

7. The process according to claim 5, wherein the acidic solution has a concentration of 0.01 to 4M.

8. The process according to claim 5, wherein the acidic solution comprises at least a salt with a proper concentration.

9. The process according to claim 5, wherein the salt has a proper concentration of 0.01 to 4M of sodium chloride.

10. The process according to claim 5, the process further comprising steps of freezing the connective tissue treated with an acidic solution, wherein the step of freezing is carried out at a proper temperature with a proper temperature reduction rate, and lyophilizing said connective tissue effective for preparing the porous collagen matrix.

11. A process for preparing a porous collagen matrix from connective tissue, the process comprising steps of treating connective tissue with an acidic solution followed by a solution of hydrogen peroxide, freezing said connective tissue at a proper temperature under a proper temperature reduction rate, and lyophilizing said connective tissue effective for preparing the porous collagen matrix.

12. The process according to claim 11, wherein the solution of hydrogen peroxide has a concentration between 0.1 and 10%.

13. The process according to claim 11, wherein the connective tissue is treated at a temperature ranging from 4 to 60.

14. The process according to claim 11, wherein the acidic solution is selected from a group consisting of peracetic acid, hydrochloric acid, oxalic acid, acetic acid and sulfuric acid.

15. The process according to claim 11, wherein the treatment with an acidic solution is divided into two steps, a first step comprising treating the connective tissue with an acetic acid solution of 0.1 to 6M and a second step comprising treating the connective tissue with an acetic acid solution of 0.01 to 2M.

16. The process according to claim 15, wherein the acetic acid solution of 0.01 to 2M comprises at least a salt.

17. The process according to claim 16, wherein the salt comprises sodium chloride of 0.01 to 4M.
18. The process according to claim 11, the process further comprising a step of treating the connective tissue with a solution containing SDS (sodium dodecyl sulphate).

19. The process according to claim 18, wherein the solution contains 0.01 to 10% of SDS.

20. The process according to claim 19, wherein the solution contains a chelating agent.

21. The process according to claim 20, wherein the chelating agent is EDTA (ethylene diamine tetra-acetic acid) with a concentration of 0.01 to 100 mM.

22. A porous collagen matrix prepared by the process according to claim 1.

23. A porous collagen matrix prepared by the process according to claim 4.

24. A porous collagen matrix prepared by the process according to claim 5.

25. A porous collagen matrix prepared by the process according to claim 7.

26. A porous collagen matrix prepared by the process according to claim 9.

27. A porous collagen matrix prepared by the process according to claim 10.

28. A porous collagen matrix prepared by the process according to claim 11.

29. A porous collagen matrix prepared by the process according to claim 15.

30. A porous collagen matrix prepared by the process according to claim 17.

31. A porous collagen matrix prepared by the process according to claim 20.

32. A porous collagen matrix prepared by the process according to claim 22.