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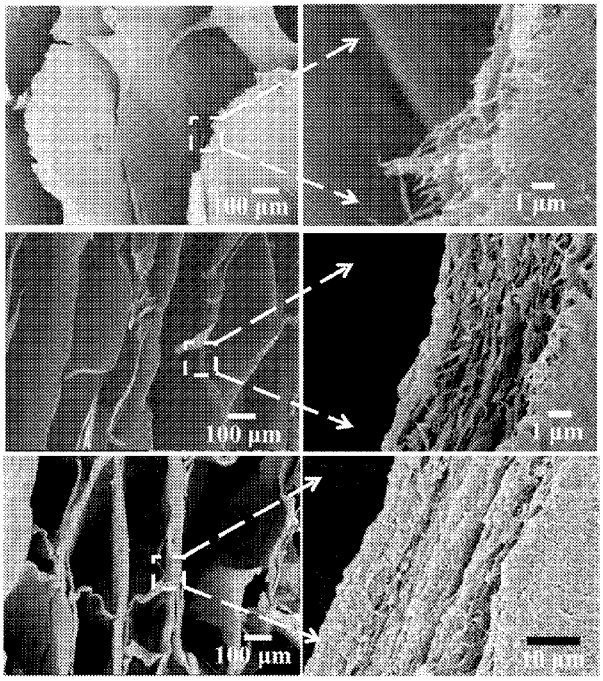
[Continued on next page]

(54) Title: BIOMIMETIC SCAFFOLD FOR BONE REGENERATION

Figure 2



(57) Abstract: Described herein are novel methods of producing collagen-apatite (Col-Ap) scaffolds that exhibit a unique, anisotropic multi-level lamellar structure in which nano and submicron pores in each lamellae and macro pores are co-aligned.

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BIOMIMETIC SCAFFOLD FOR BONE REGENERATION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application 61/685,689 filed on March 22, 2012, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH & DEVELOPMENT

[0001] This invention was made with government support under Grant # CBET 1133883 awarded by the National Science Foundation (NSF) and under Grant # AR 059962 awarded by the National Institutes of Health (NIH). The government has certain rights in the invention.

FIELD OF THE DISCLOSURE

[0002] The present disclosure is in the field of compositions and methods for tissue engineering, and more specifically for bone regeneration.

BACKGROUND

[0003] Collagen-apatite (Col-Ap) composites resembling the composition of natural bone have been studied extensively and are considered as promising bone tissue engineering materials. Apatite exhibits good biocompatibility, osteoconductivity and bone-bonding ability, but its brittleness, rigidity, and low degradation rate limit its usefulness in broader applications. Collagen, the most abundant protein of extracellular matrix, is chemotactic to fibroblasts. It shows high affinity to cells and good resorbability in vivo. Nevertheless, its poor mechanical strength has restricted its usage in load-bearing applications. By adding apatite to collagen, the mechanical properties of the resulting composite could increase substantially. It was also reported that Col-Ap composite scaffolds demonstrate better osteoconductive properties and higher levels of osteogenic gene expression than non-mineralized collagen scaffolds.

[0004] Many approaches to the production of Col-Ap scaffolds have been developed, however, what is needed are Col-Ap scaffold of defined structure and methods of making the structures.

BRIEF SUMMARY

[0005] In one aspect, included herein is a method of producing a structural protein-calcium phosphate scaffold with an anisotropic lamellar pore structure, comprising providing a structural protein-calcium phosphate hydrogel; compressing the structural protein-calcium phosphate hydrogel to increase the structural protein density in the hydrogel; freezing the hydrogel with a temperature gradient in a transverse direction across the hydrogel; and drying the frozen hydrogel to produce the structural protein-calcium phosphate scaffold with an aligned lamellar structure. In specific aspects, the structural protein is collagen and the calcium phosphate is hydroxylapatite. Also included are the products of the processes described herein.

[0006] In another aspect, included herein is a biomimetic structural protein-calcium phosphate scaffold with an anisotropic lamellar pore structure, wherein at the macro-level, the scaffold comprises an anisotropic lamellar pore structure and a co-aligned macro-pore size of 10 to 350 micrometers, and at the micro-level each lamella of the lamellar structure comprises uniaxial aligned layers of structural protein fibers mineralized with the calcium phosphate having a micro-pore size of less than 1 micrometer.

[0007] In a further aspect, a method of bone repair comprises contacting the structural protein-calcium phosphate scaffold with an anisotropic lamellar pore structure with osteoprogenitor cells, bone marrow cells, or both, under conditions suitable to repair bone.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Referring now to the drawings wherein like elements are numbered alike in several FIGURES:

[0009] Figure 1 (a, c-e) show FESEM images of a collagen-apatite scaffold. (b) MicroCT of 3-dimensional reconstruction of a collagen-apatite scaffold. The scaffold possesses a multi-level lamellar structure having (a and b) co-aligned macro-pores, (c) nano and submicro-pores, and (d and e) a biomimetic surface containing mineralized fibril bundles. (f) TEM image demonstrating needle-like apatite crystallites throughout the cross-section of collagen fibers similar to those found in natural bone, (g) High magnification of selected area electron diffraction patterns exhibits typical rings of low crystalline apatite structure. (The Miller indices for each corresponding ring are labeled)

[0010] Figure 2 shows FESEM images of cross-section parallel to the freezing direction for scaffolds fabricated by freezing the hydrogel at a constant freezing temperature of -25 °C, but with a collagen density of (a) 2.5g/L, (b) 3.9g/L and (c) 10.5g/L.

[0011] Figure 3 shows representative unconfined compressive stress-strain curves of collagen-apatite scaffolds.

[0012] Figure 4 shows a schematic presentation of a method to prepare a collagen-apatite scaffold: a) fresh hydrogel, b) compression of the hydrogel and c) freezing of the compressed hydrogel. C_0: initial collagen concentration in the hydrogel, Ct: collagen concentration in the hydrogel after self-compression for a certain period of time (t), C_f: collagen concentration in the frozen gel.

[0013] The above-described and other features will be appreciated and understood by those skilled in the art from the following detailed description, drawings, and appended claims.

DETAILED DESCRIPTION

[0014] Described herein are novel methods of producing scaffolds such as collagen-apatite (Col-Ap) scaffolds that exhibit a unique multi-level lamellar structure in which nano and submicron pores in each lamella and macro-pores are co-aligned. This multi-level lamellar structure leads to a higher active surface area, improved permeability to oxygen and nutrients, and faster removal of metabolic waste compared to the conventional uniaxial pore structure. Another unique property of the scaffold is the combination of a long-range ordered surface morphology and 3-D porous structure. In one aspect, the surface of the scaffold comprises mineralized collagen fibrils with a length of over a few millimeters. In this aspect, the mineralized collagen fibrils are further organized into big bundles, the diameter of which can be tailored by gelation conditions in terms of temperature and initial collagen concentration.

[0015] The novel structural protein-calcium phosphate scaffold with an anisotropic lamellar pore structure has a unique macro and micro-level structure. At the macro-level, the scaffold exhibits an anisotropic lamellar pore structure and a co-aligned pore size of 10 to 350 micrometers. At the micro-level, each lamella of the lamellar structure includes uniaxial aligned layers of structural protein fibers mineralized with the calcium phosphate having a micro pore size of less than 1 micrometer. The porosity of the scaffolds can be greater than 85%. In one embodiment, the scaffold protein is collagen and the calcium phosphate is hydroxylapatite. In another embodiment, the wall thickness of the lamellar layers is 2 to 30 micrometers. An advantage of the methods described herein is that the wall thickness is tunable depending on the conditions used to produce the scaffold. In yet another embodiment, the scaffold exhibits fewer visual bridges between the lamellar layers than

observed in previous structures. The basic building block of the multi-level lamellar structure described herein is a network of long and interconnected mineralized collagen fibers. The length of each fiber can be extended to a few centimeters, such as greater than 5 centimeters.

[0016] In one aspect, a method of producing a structural protein-calcium phosphate scaffold (e.g., collagen-hydroxylapatite, Col-Ap) with an anisotropic lamellar pore structure comprises providing a structural protein-calcium phosphate hydrogel; compressing the structural protein-calcium phosphate hydrogel to increase the structural protein density in the hydrogel; freezing the hydrogel with a temperature gradient in a transverse direction across the hydrogel; and drying the frozen hydrogel to produce the structural protein-calcium phosphate scaffold with an aligned lamellar structure.

[0017] Compression of the hydrogel raises the chemical potential of the hydrogel and causes water to be exuded from the hydrogel. Without being held to theory, it is believed that in the compression process, the fluid leaving surface acts as a filter along which compacted lamellae of mineralized collagen fibrils are aligned.

[0018] In one embodiment, compression is self-compression performed, for example, temperature of 4 to 45°C and a time of 5 minutes to 5 hours. Self-compression is performed, for example, by removing the hydrogel from a container and allowing it to undergo unconfined self-compression. As used herein, unconfined self-compression means uniaxial loading without lateral confining pressures. Alternatively, compression can be performed by placing a weight on the hydrogel. A compressive load can be applied, for example, by placing a piece of thick glass/metal plate on top of the hydrogel for 5 minutes to 5 hours. The collagen concentration in the hydrogel can be tailored by the mass of the plate. In one aspect, compression leads to an increase of the collagen concentration in the hydrogel thereby increasing the Young's modulus of the freeze dried hydrogel by 5-30-fold.

[0019] After compression, the hydrogel is frozen with a temperature gradient in a transverse direction across the hydrogel. Freezing can be accomplished in a mold that allows for control of the temperature in the transverse direction. An exemplary mold is a copper mold with its cover and bottom made from Teflon®. As the temperature gradient advances, the advancing ice front causes diminishing liquid, pushing the structural protein-calcium phosphate to higher concentrations. The ice front applies shear stress on the network of the hydrogel which increase the degree of the alignment of mineralized structural protein fibril arrays in each lamella.

[0020] In practice, freezing with a temperature gradient in a transverse direction across the hydrogel can be accomplished using a cylindrical mold designed as follows. The

top and bottom of the cylindrical mold are made from heat insulating materials (Teflon®) to reduce heat transfer from the surrounding environment to the gel. The wall of the mold is made from materials with high thermal conductivity (copper) to create the thermal gradient transversely form the edges to the center of the gel.

[0021] In one embodiment, the freezing temperature is -197°C to -10°C. In another embodiment freezing takes place over 0.5 to 4 hours. In one aspect, the cooling rate during freezing is 1 to 40°C/min.

[0022] Once the compressed hydrogel is frozen, it is dried under vacuum conditions, such as in a freeze-drying apparatus, to produce the structural protein-calcium phosphate scaffold with an aligned lamellar structure. In one aspect, the drying temperature is -10 to -80 °C and the pressure in the vacuum chamber is 0.2 to 3 mbr, the drying time is 10 to 120 hours. In one embodiment, after drying, the structural protein-calcium phosphate scaffold with an anisotropic lamellar pore structure has a structural protein content of 40 to 99 wt% or higher.

[0023] In one aspect, a structural protein-calcium phosphate hydrogel is prepared in a one-step process. The method involves preparing an aqueous system containing water, Ca^{2+}, HPO_4^{2-}, structural protein (e.g., a collagen such as collagen type I), a weak acid (e.g., acetic acid, and the like) and a buffer system; and optionally one or more of the following ions: Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-}, HCO_3^-; wherein the aqueous system has an initial pH of about 6 to about 8. The aqueous system is allowed to stand, for example at a temperature of about 4°C to about 45°C, for a period of greater than one hour, specifically greater than 10 hours, to form a hydrogel. The gel is optionally crosslinked, isolated.

[0024] The structural protein includes known structural protein such as collagens, elastin, and keratins, specifically collagen, and more specifically acetic acid soluble collagen, including Types I, II, III, and V, and yet more specifically collagen Type I. In one embodiment, the concentration of structural protein in the aqueous system is 1 g/L to 10.0 g/L.

[0025] There is no particular limitation as to the source of the structural protein. The structural protein may be obtained from commercial sources or extracted from natural sources using procedures well known in the art.

[0026] The aqueous system used to prepare the structural protein-calcium phosphate hydrogel generally comprises the following inorganic ions: Ca^{2+} and HPO_4^{2-}; and optionally one or more of the following ions: Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-}, HCO_3^-. The aqueous system can be prepared by dissolving, in an aqueous solvent, salt that when disassociated will result

in the particular ions Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, SO_4^{2-}, HPO_4^{2-} and HCO_3^{-}. The aqueous solvent can be deionized and purified water. Exemplary salts include those that result in an aqueous solution of the desired ions, for example, alkali metal halides, alkaline earth metal halides, alkali metal hydrogen carbonates, alkali metal phosphates, and alkali metal sulfates. Specific salts include NaCl, KCl, K_2HPO_4, $MgCl_2$, Na_2SO_4, $CaCl_2$ and $NaHCO_3$.

[0027] The particular concentrations of each of the above-described ions initially present in the aqueous system can be as follows: Ca^{2+} at about 0.1 to about 30.0 mM, specifically about 1 to about 15.0 mM, and more specifically about 5 to about 10.0 mM; Mg^{2+} at about 0 to about 10.0 mM, specifically about 0.5 to about 5.0 mM, and more specifically about 1.0 to about 3 mM; Na^{+} at about 0 to about 300.0 mM, specifically about 50.0 to about 200.0 mM, and more specifically about 80.0 to about 150.0 mM; K^{+} at about 0 to about 20.0 mM, specifically about 1.0 to about 15.0 mM, and more specifically about 4.0 to about 10.0 mM; Cl^{-} at about 0 to about 300.0 mM, specifically about 50.0 to about 200.0 mM, and more specifically about 80.0 to about 150.0 mM; SO_4^{2-} at about 0 to about 5.0 mM, specifically about 0 to about 1.5 mM, and more specifically about 0 to about 0.6 mM; HPO_4^{2-} at about 0.05 to about 20.0 mM, specifically about 0.1 to about 10.0 mM, and more specifically about 0.5 to about 5.0 mM; and HCO_3^{-} at about 0 to about 50.0 mM, specifically about 5.0 to about 30.0 mM, and more specifically about 10.0 to about 20.0 mM.

[0028] An additional component present in the aqueous system used to prepare the structural protein-calcium phosphate hydrogel is a buffer system. The buffer system can contain HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid or N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Molecular formula: $C_8H_{17}N_2SO_3$; CAS No: 7365-45-9) and an alkali metal hydrogen carbonate (e.g. $NaHCO_3$, $KHCO_3$, etc.) which are added to the aqueous system in amounts to substantially stabilize the aqueous system. The concentration of HEPES present in the aqueous system can be at about 5.0 grams per liter (g/L) to about 80.0 g/L, specifically about 10.0 g/L to about 60.0 g/L, and more specifically about 12.0 g/L to about 48.0 g/L.

[0029] Additional buffer systems include tris-hydroxymethyl aminomethane (TRIS), HEPES salts, piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), PIPES salts, combinations of the foregoing with an alkali metal carbonate, and combinations thereof.

[0030] The aqueous system may optionally contain additional ionic components such as silicate, strontium, zinc, silver, fluoride, combinations thereof, and the like.

[0031] The weak acid present in the aqueous system used to prepare structural protein-calcium phosphate hydrogel is an acid with a pKa of about 3.5 to about 5.5.

Exemplary acids include organic acids, specifically alkyl carboxylic acids such as acetic acid, propionic acid, and the like.

[0032] The aqueous system can have an initial pH of about 6 to about 8, specifically about 7 to about 7.5.

[0033] Various crosslinking agents, such as a carbodiimide, can be used to crosslink the structural protein in the hydrogel. Exemplary crosslinking agents include glutaraldehyde, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride optionally in combination with *N*-hydroxysuccinimide or *N*-hydroxysulfosuccinimide; dimethyl suberimidate, bis(sulfosuccinimidyl)suberate (BS^3), 3,3´-dithiobis(sulfosuccinimidylpropionate) (DTSSP), sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC), dithiobis(succinimidyl)propionate (DSP), sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate, and the like. In specific embodiment, the amount of crosslinking agent used is about 0.1 to about 0.4 M, specifically about 0.2 to about 0.3 M. The hydrogel can be crosslinked before or after drying.

[0034] In one embodiment, a method of forming a structural protein-calcium phosphate hydrogel comprises forming an aqueous system comprising a structural protein, a weak acid, water, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}, SO_4^{2-}, HPO_4^{2-}, HCO_3^{-} and a buffer system, wherein the aqueous system has an initial pH of about 6 to about 8, in container; and allowing the structural protein-calcium phosphate hydrogel to form in the container.

[0035] The resulting hydrogel generally contains hydroxylapatite, but can also be other types of calcium phosphate. Exemplary calcium phosphate minerals include $Ca_5(PO_4)_{3-x}(OH)_{1-y}(CO_3)_{x+y}$, $Ca_5(PO_4)_3(OH)$, $Ca_3(PO_4)_2$, $CaHPO_4$, $Ca(H_2PO_4)_2$, and the like.

[0036] In one embodiment, the novel scaffolds described herein further comprise a drug such as a drug that can improve the bone-regeneration properties of the scaffold. Drugs can be incorporated into the scaffold by adding drug to m-SBF or depositing the drug at the surface of freeze-dried scaffolds. Exemplary drugs for incorporation into the scaffolds include antibiotics and antiseptics (e.g., gentamicin, tetracycline, minocycline), vitamins (e.g., riboflavine), and the like.

[0037] In one aspect, the novel scaffold described herein is used in methods of bone repair. In one aspect, a method of bone repair comprises contacting the novel scaffold described herein with osteoprogenitor cells, bone marrow cells, or both, under conditions suitable to repair bone. Contacting can be *in vitro* or *in vivo* in a host. Bone repair can include new bone formation, bone-redistribution, bone-host integration, scaffold degradation, or a combination thereof.

[0038] In one aspect, the novel collagen-apatite scaffolds can be tested in a double-hole mouse calvarial model for evaluating new bone formation. A series of transgenic mice harboring GFP reporters that mark different levels of osteoprogenitor lineage differentiation have been developed. The pOBCol3.6GFP transgene is activated at an early stage of preosteoblast differentiation and continues being expressed strongly in osteoblasts lining on new bone surfaces. The development of transgenic mice harboring type I collagen GFP reporters and the ability to reserve the fluorescent signal during histological processing make it possible to use transgenic mice to evaluate host/donor cell behavior during cell-based bone healing. In this study, multiple types of cells from different tissue sources, including both osteoprogenitor cells (OPCs) and bone marrow cells (BMCs), are used in combination with scaffolds for bone regeneration. New bone formation can be observed by microscopy techniques either with single cell type or multiple cell types. Without being held to theory, the amount of new bone formed, bone distribution, new bone-host bone integration, and scaffold degradation may be distinctly different among these groups. According to the results from fluorescence imaging analysis and H&E histology, new bone formation can be confirmed.

[0039] The invention is further illustrated by the following non-limiting examples.

Example 1: Preparation of and characterization of Col-Ap hydrogel

[0040] The biomimetic collagen-apatite hydrogel was synthesized using a collagen containing modified simulated body fluid (m-SBF; 109.5 mM Na^+, 6 mM K^+, 1.5 mM Mg^{2+}, 7.5 mM Ca^{2+}, 110.0 mM Cl^-, 17.5 mM HCO_3^{2-}, 3.0 mM HPO_4^{2-}, 50 mM HEPES). The concentration of collagen in m-SBF was adjusted to 2 g/L to achieve an apatite content of 35% in the scaffold (wt%). The collagen concentration in the m-SBF can be tailored to provide scaffolds with different apatite contents. The pH of the m-SBF solution was adjusted to 7 by addition of HEPES (4-(2-hydroxyapatiteethyl)-1-piperazineethanesulfonic acid) and NaOH. The collagen-apatite hydrogel was prepared using a two temperature process. In this process, the solution was incubated in a sealed vial at 25°C for 1 h, the temperature was then increased at a rate of 0.5 °C/min to 40°C and left at 40°C for 22.5 h.

[0041] The collagen-apatite hydrogel was allowed to undergo unconfined self-compression at room temperature for different time periods. Because the compression force applied by self-gravity raised the chemical potential of water inside the hydrogel, water was exuded from the gel. The mineralized collagen fibrillar density of the gel can be easily

controlled by changing the self-compression time. External compression force can also be applied, for example, by placing a weight on top of the hydrogel.

[0042] The collagen-apatite gel was made in a custom-made mold which can control the temperature gradient in the transverse direction. The top and bottom of the mold were made from Teflon® to reduce heat transfer from the surrounding environment to the gel. The wall of the mold was made from copper to create a thermal gradient transversely form the edge to the center of the gel. The mold was placed in a chamber precooled to -10°C to -180°C. The temperature in the cold chamber was varied intending to modify the spacing between each lamella.

[0043] The as-frozen hydrogel was then lyophilized in a freeze dryer. The freeze-dried scaffolds were subsequently cross-linked with 1 wt% N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) hydrochloride for 24 h. Then scaffolds were rinsed thoroughly in distilled water, followed by rinsing with 5% glycine solution, rinsing again with water, and finally freeze-drying for a second time.

[0044] Figure 1 (a, c-e) show FESEM images of a collagen-apatite scaffold. (b) MicroCT of 3-dimensional reconstruction of a collagen-apatite scaffold. The scaffold possesses a multi-level lamellar structure having (a and b) co-aligned macro-pores, (c) nano and submicro-pores, and (d and e) a biomimetic surface containing mineralized fibril bundles. (f) TEM image demonstrating needle-like apatite crystallites throughout the cross-section of collagen fibers similar to those found in natural bone, (g) High magnification of selected area electron diffraction patterns exhibits typical rings of low crystalline apatite structure. (The Miller indices for each corresponding ring are labeled)

[0045] The scaffold exhibits a unique multi-level lamellar structure in which nano and submicron pores in each lamella and macro pores are co-aligned. This multi-level lamellar structure may lead to higher active surface area, improved permeability of oxygen and nutrients, and faster removal of metabolic waster compared to conventional uniaxial pore structure. Another unique property of the scaffold is the combination of a long-range ordered surface morphology and 3-dimensional porous structure. The surface of the scaffold is comprised of mineralized collagen fibrils with a length of over a few millimeters. The mineralized collagen fibrils further organized into big bundles and the diameter of which can be tailored by gelation conditions in terms of temperature and initial collagen concentration. Figure 2 shows FESEM images of cross-section parallel to the freezing direction for scaffolds fabricated by freezing the hydrogel at a constant freezing temperature of -25°C, but with a collagen density of (a) 2.5g/L, (b) 3.9g/L and (c) 10.5g/L.

[0046] To the best of our knowledge, this is the first report of a process to engineer three dimensional collagen-apatite hybrid scaffolds with controllable pore size and pore orientation from nano-scale to the macro-scale. With an increase of collagen density from 2.5 to 10.5 g/L, lamellar spacing decreased from 343.5±32.7 μm to 142.9±40.1 μm and wall thickness increased from 3.6±1.0 μm to 23.2±10.2 μm.

Table 1 Compression modulus of collagen-apatite scaffolds

Collagen density in the hydrogel (g/L)	Compression modulus (kPa)-X	Compression modulus (kPa)-Z
2.5	222.6 ± 69.1	59.5 ± 10.3
3.9	2912.5± 802.0	149.9 ± 25.6

[0047] Figure 3 shows representative unconfined compressive stress-strain curves of collagen-apatite scaffolds. The stress-strain curve of the scaffolds under compression along the pore direction was similar to cancellous bone. The increase of collagen-apatite hydrogel density leads to an increase in the compression modulus (Table 1). Therefore, the mechanical strength of the scaffold could be greatly improved by increasing the initial collagen fibril density within the hydrogel. Uniaxial tensile test performed on collagen and collagen-apatite scaffold indicated that the addition of apatite increases the Young's modulus of collagen scaffold (Table 2).

Table 2 Young's modulus of collagen and collagen-apatite scaffold

Sample	Collagen density in the hydrogel (g/L)	Young's modulus (MPa)
Collagen	3.9	13.9± 7.4
Collagen-apatite	3.9	263.8± 72.1

[0048] Figure 4 is a schematic presentation of a method including gelation, compression and unidirectional freezing to prepare the collagen-apatite scaffolds with a multi-level lamellar structure. C_0: initial collagen concentration in the hydrogel, C_t: collagen concentration in the hydrogel after self-compression for a certain period of time (t), C_f: collagen concentration in the frozen gel.

[0049] A hydrogel with increased collagen concentration was produced using a simple self-compression method and the resulting C_t is time dependent. The fresh hydrogel with a soft texture is mechanically unstable, because the compression force applied by self-gravity raised the chemical potential of water inside the hydrogel. As a result, water was exuded from the gel. The collagen fibrillar density of the gel can be easily controlled by the self-compression time (t). During the above unconfined self-compression process, the main fluid leaving surface acts as a filter along which compacted lamellae of mineralized collagen fibrils were aligned. Then the compressed hydrogel with a fibrillar concentration of C_t was frozen in a mold that can control the temperature gradient in the transverse direction. The mineralized collagen fibrils were pushed by the advancing ice front therefore the Col-Ap density in the diminishing liquid increased to higher concentration (C_f). The ice front applied shear stress on the network of hydrogel thereby may further increase the degree of the alignment of mineralized collagen fibril arrays in each lamellae. The dendritic like surface topography in the solidification direction confirmed that aligned macro pores were created by uni-directionally freezing the hydrogel at different cooling rates from 1°C/min to 40 °C/min using the house made mold.

[0050] Advantageously, the methods to produce the novel scaffold structures disclosed herein allow for one-step production of the hydrogel, allowing a fast hydrogel production. The cost of set-up to produce the scaffolds is low, and the processes are easy to scale-up. The controllable porosity should allow for drug uptake into the scaffolds. These novel scaffolds are expected to have particular utility in tissue engineering applications.

[0051] The use of the terms “a” and “an” and “the” and similar referents (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms first, second etc. as used herein are not meant to denote any particular ordering, but simply for convenience to denote a plurality of, for example, layers. The terms “comprising”, “having”, “including”, and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to”) unless otherwise noted. Recitation of ranges of values are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. The endpoints of all ranges are included within the range and independently combinable. All methods described herein can be performed in a suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary

language (e.g., "such as"), is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention as used herein.

[0052] While the invention has been described with reference to a preferred embodiment, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the scope of the invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from essential scope thereof. Therefore, it is intended that the invention not be limited to the particular embodiment disclosed as the best mode contemplated for carrying out this invention, but that the invention will include all embodiments falling within the scope of the appended claims.

CLAIMS

1. A method of producing a structural protein-calcium phosphate scaffold with an anisotropic lamellar pore structure, comprising

providing a structural protein-calcium phosphate hydrogel;

compressing the structural protein-calcium phosphate hydrogel to increase the structural protein density in the hydrogel;

freezing the hydrogel with a temperature gradient in a transverse direction across the hydrogel; and

drying the frozen hydrogel to produce the structural protein-calcium phosphate scaffold with an aligned lamellar structure.

2. The method of claim 1, wherein compression is unconfined self-compression.

3. The method of claim 2, wherein self-compression is performed at a temperature of 4 to 45°C.

4. The method of claim 1, wherein the freezing temperature is -197°C to -10°C.

5. The method of claim 4, wherein the freezing takes place over 0.5 to 4 hours.

6. The method of claim 1, wherein the structural protein is collagen Type I, II, III, or V.

7. The method of claim 6, wherein the calcium phosphate is hydroxylapatite.

8. The method of claim 1, wherein the structural protein-calcium phosphate scaffold with an anisotropic lamellar pore structure has a structural protein content of 40 to 99 wt%.

9. The method of claim 1, further comprising crosslinking the structural protein-calcium phosphate scaffold after drying.

10. The method of claim 1, wherein the structural protein-calcium phosphate hydrogel is formed by

forming an aqueous system comprising a structural protein, a weak acid, water, Ca^{2+}, HPO_4^{2-}, a buffer system, and optionally one or more of Mg^{2+}, Na^+, K^+, Cl^-, SO_4^{2-}; or HCO_3^-; wherein the aqueous system has an initial pH of about 6.0 to about 8.0, in a container; and

allowing the structural protein-calcium phosphate hydrogel to form in the container at a temperature of 4-45°C and a time of greater than one hour.

11. The method of claim 10, wherein the hydrogel is formed in a period of greater than 10 hours.

12. The method of claim 10, wherein

the structural protein is collagen Type I present in an amount of 1 g/L to 10.0 g/L of

the aqueous system;

Ca^{2+} is present in an amount of 0.1 to 30.0 mM;

Mg^{2+} is present in an amount of 0.05 to 10.0 mM;

Na^{+} is present in an amount of 5.0 to 300.0 mM;

K^{+} is present in an amount of 0.1 to 20.0 mM;

Cl^{-} is present in an amount of 5.0 to 300.0 mM;

SO_4^{2-} is present in an amount of 0 to 5.0 mM;

HPO_4^{2-} is present in an amount of 0.05 to 20.0 mM; and

HCO_3^{-} is present in an amount of 0.5 to 50.0 mM.

13. The product of the process of claim 1.

14. A biomimetic structural protein-calcium phosphate scaffold with an anisotropic lamellar pore structure,

wherein at the macro-level, the scaffold comprises an anisotropic lamellar pore structure and a co-aligned macro-pore size of 10 to 350 micrometers, and

at the micro-level each lamella of the lamellar structure comprises uniaxial aligned layers of structural protein fibers mineralized with the calcium phosphate having a pore size of less than 1 micrometer.

15. The biomimetic structural protein-calcium phosphate scaffold of claim 14, wherein the scaffold protein is collagen and the calcium phosphate is hydroxyl apatite.

16. The biomimetic structural protein-calcium phosphate scaffold of claim 15, wherein the wall thickness of the lamellar layers is 2 to 30 micrometers.

17. The biomimetic structural protein-calcium phosphate scaffold of claim 14, further comprising a drug.

18. A method of bone repair, comprising contacting the scaffold of claim 14 with osteoprogenitor cells, bone marrow cells, or both, under conditions suitable to repair bone.

19. The method of claim 18, wherein bone repair comprises new bone formation, bone-redistribution, bone-host integration, scaffold degradation, or a combination thereof.

Figure 1

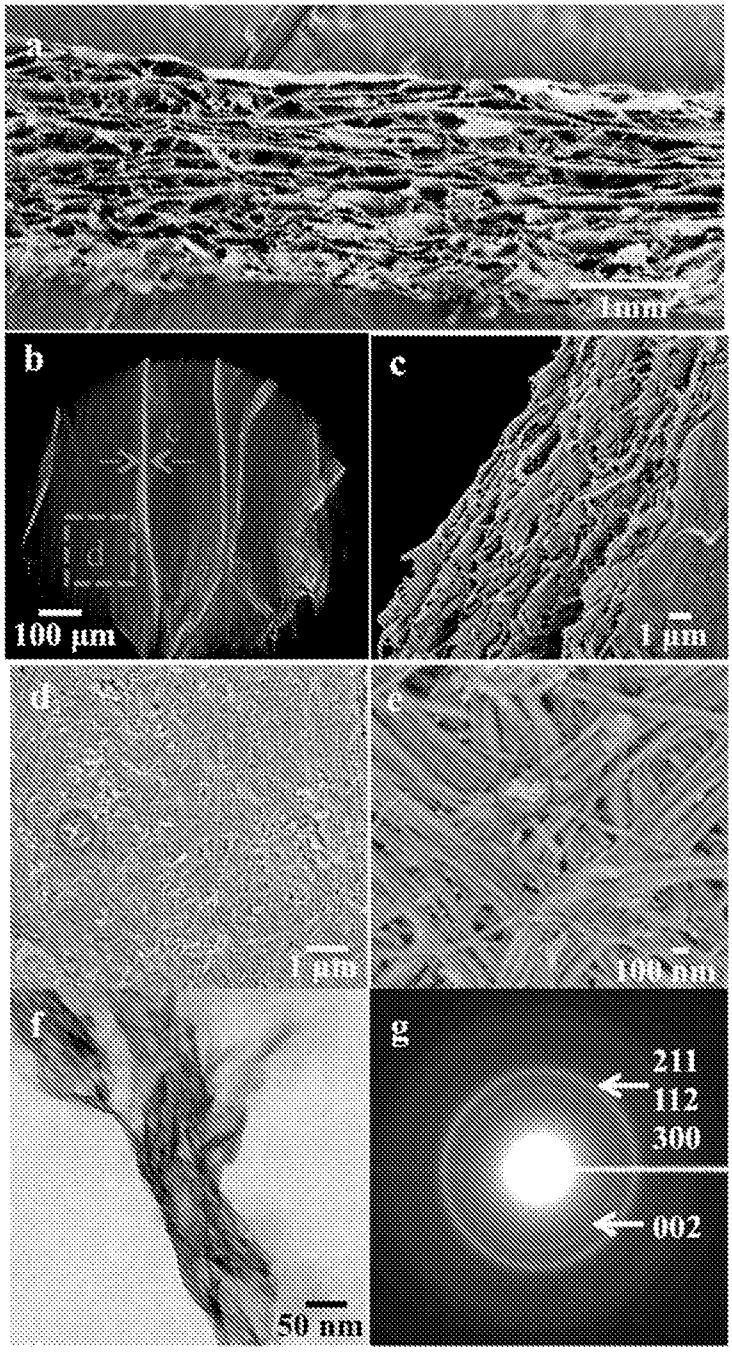


Figure 2

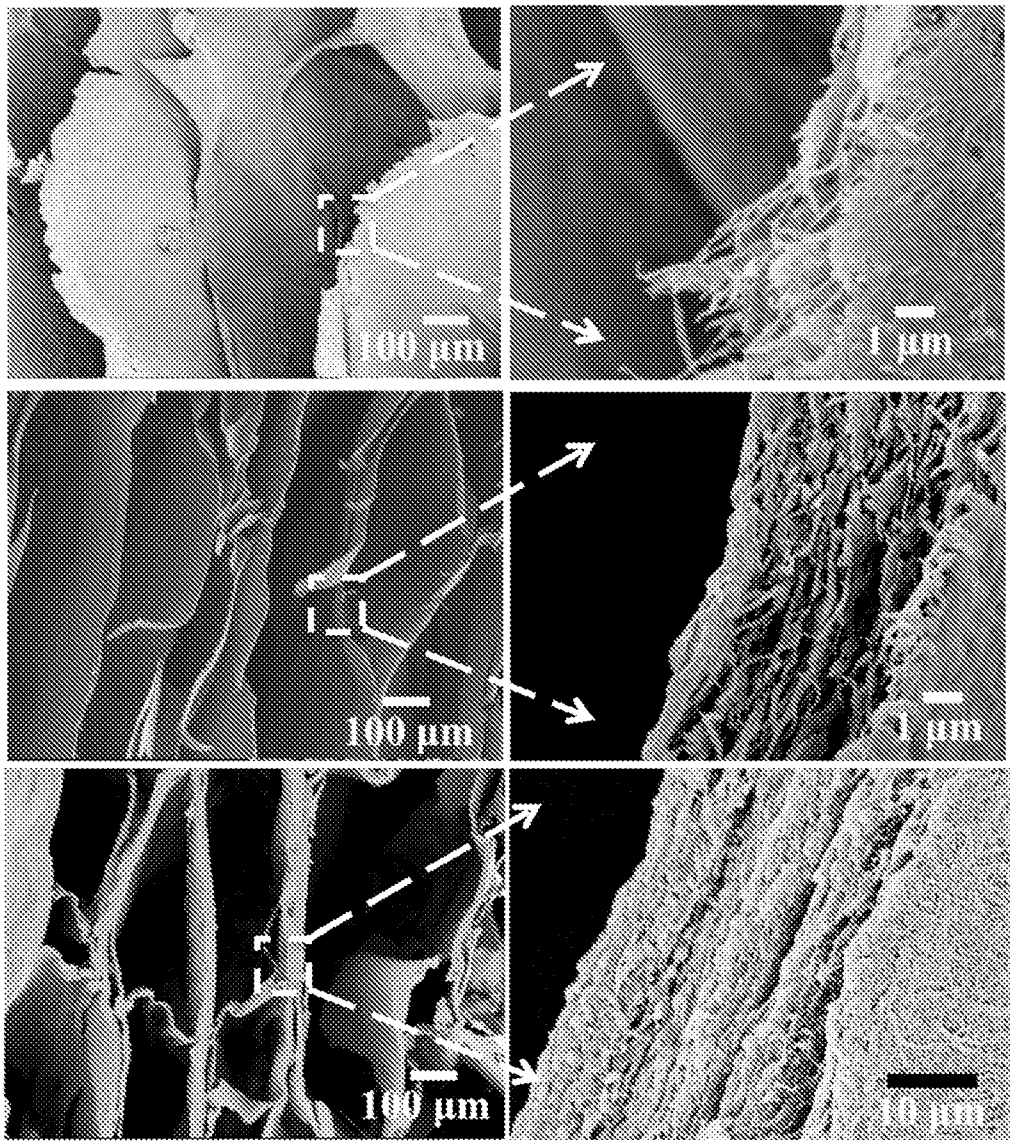


Figure 3

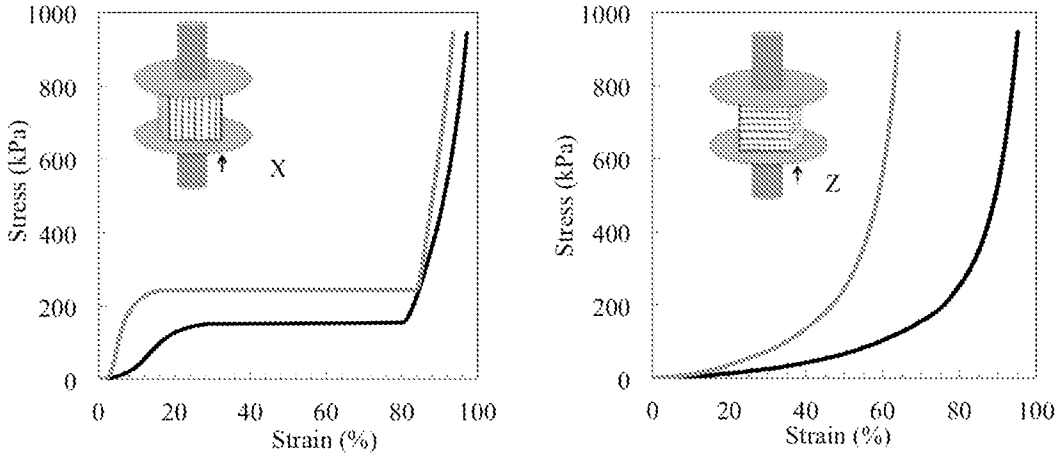
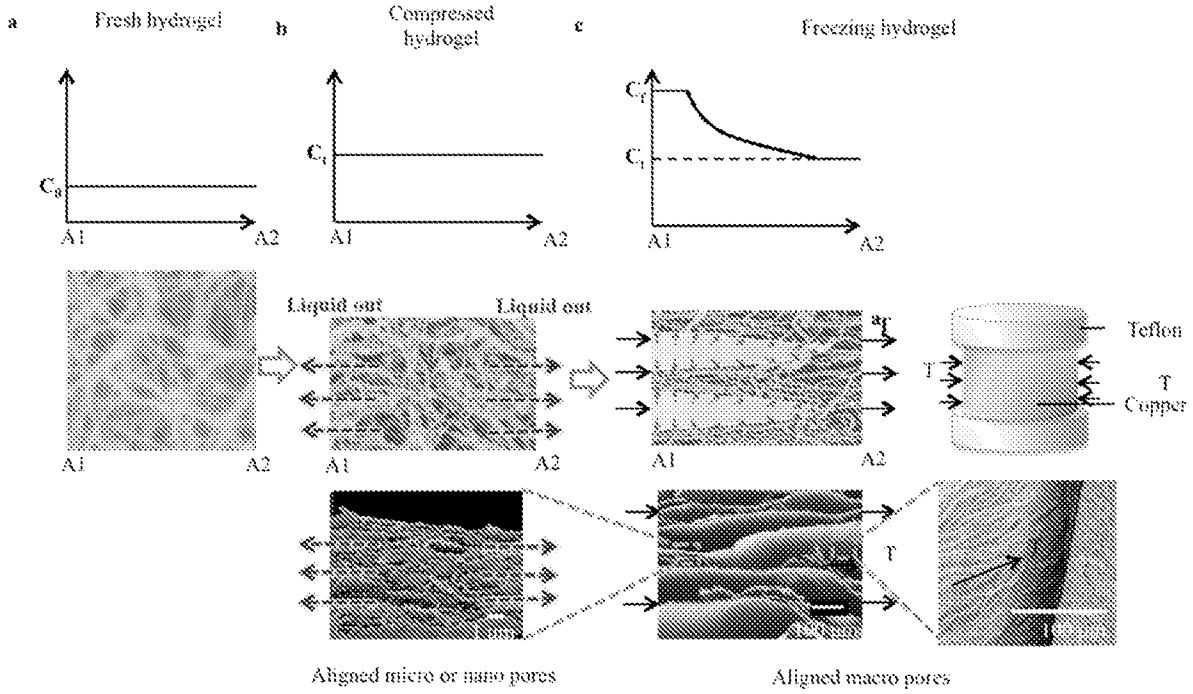


Figure 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/033443

A. CLASSIFICATION OF SUBJECT MATTER

A61L 27/56(2006.01)i, A61L 27/24(2006.01)i, A61L 27/22(2006.01)i, A61L 27/52(2006.01)i, A61F 2/28(2006.01)i

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 27/56; A61F 2/28; A61F 2/02; A61F 2/00; A61L 27/00; C07K 14/78; A61L 27/46; A61L 27/24; A61L 27/22; A61L 27/52

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: protein-calcium phosphate scaffold, anisotropic, lamella, pore, collagen, hydroxylapatite, compressing, freezing, temperature gradient

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2007-055431 A1 (SEOUL NATIONAL UNIVERSITY INDUSTRY FOUNDATION) 18 May 2007 See abstract; paragraphs [76], [77]; claims 1, 2, 6; figures 3(A)-3(E).	1-9,13
A		10-12,14-17
Y	US 2004-0259972 A1 (RINGEISEN et al.) 23 December 2004 See abstract; paragraph [0055]; claims 1, 4, 6, 7, 9; figures 5A-6.	1-9,13,15-17
A		10-12,14
X	LANDI et al. `Porous hydroxyapatite/gelatine scaffolds with ice-designed channel-like porosity for biomedical applications` Acta Biomaterialia, Vol.4, pp.1620-1626 (6 June 2008) See abstract; pages 1621, 1623; figure 3.	14
Y		15-17
A		1-13
A	US 2002-0018797 A1 (CUI et al.) 14 February 2002 See abstract; claims 1, 2, 4; figures 1A-3.	1-17
A	US 2009-0149634 A1 (SHOJI et al.) 11 June 2009 See abstract; claims 1, 6.	1-17

☐ Further documents are listed in the continuation of Box C.

☒ 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 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
08 July 2013 (08.07.2013)	**09 July 2013 (09.07.2013)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/033443

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 18,19
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 18, 19 pertain to methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/033443

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007-055431 A1	18/05/2007	EP 1951331 A1	06/08/2008
		EP 1951331 A4	24/03/2010
		US 2012-114763 A1	10/05/2012
US 2004-0259972 A1	23/12/2004	CA 2532829 A1	29/12/2004
		EP 1638621 A1	29/03/2006
		EP 2305320 A2	06/04/2011
		EP 2305320 A3	13/04/2011
		US 2006-0002980 A1	05/01/2006
		US 2007-0202148 A1	30/08/2007
		US 2011-0133368 A1	09/06/2011
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		US 6974862 B2	13/12/2005
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		US 7910690 B2	22/03/2011
		US 8188229 B2	29/05/2012
		WO 2004-112854 A1	29/12/2004
US 2002-0018797 A1	14/02/2002	CN 1106861 C	30/04/2003
		CN 1272383 A	08/11/2000
		US 6887488 B2	03/05/2005
US 2009-0149634 A1	11/06/2009	JP 2009-132601 A	18/06/2009