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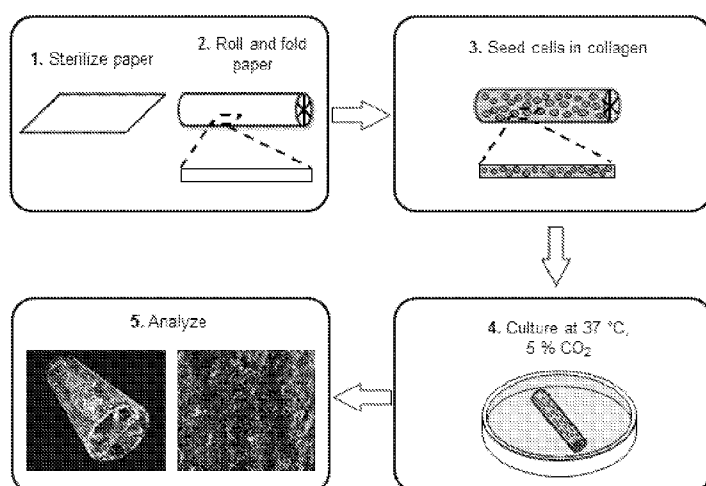
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[Continued on next page]

(54) Title: BIOMINERALIZATION ON PAPER SCAFFOLDS

Figure 1A



(57) **Abstract:** Bone-mimetic microstructures are formed within the paper scaffolds. Centimeter scale paper origami scaffolds are seeded with osteoblasts and cultured to induce formation of mineral deposits. The paper-based platform is a promising system to study mechanisms of biomineralization, repair mineralized tissues, understand healing and regeneration of bone, develop therapeutic approaches when mineralization is impaired, and understand the fundamental processes in biomineralization events.



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BIOMINERALIZATION ON PAPER SCAFFOLDS

RELATED APPLICATIONS

[0001] This application claims the benefit of priority under 35 U.S.C. §119(e) to co-pending United States Application Ser. No. 62/111,891, filed February 4, 2015, the contents of which are incorporated by reference.

INCORPORATION BY REFERENCE

[0002] All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0003] The present invention was made with United States government support under DARPA Grant No. W911NF-09-1-0044 awarded by the Department of Defense. The United States government may have certain rights in this invention.

BACKGROUND

[0004] Bone is a dense mineralized connective tissue serving mechanical functions, protecting internal organs, assisting with movement, storing minerals, and generating red and white blood cells. Bone is composed of extracellular matrix, carbonated apatite, water, and cells. Disease, degeneration, or trauma can cause bone loss in the body. While several approaches exist to repair or regenerate the bone tissue, they generally use expensive materials and complex multi-step protocols to generate cell-laden three-dimensional (3D) scaffolds. Scaffolds for bone have been fabricated using different methods, including 3D

printing, electrospinning, gas foaming, injection molding, and salt leaching. These methods require special equipment and trained personnel. The resulting constructs often times contain heterogeneously distributed cells and demonstrate diffusion limitations; therefore, it is challenging to produce clinically relevant size biocompatible constructs for formation of bone. There is, thus, a tremendous demand for inexpensive and simple platforms that can recapitulate the events that occur during biomineralization and formation of bone.

[0005] The ideal scaffold for regeneration of bone is desirably porous, biocompatible and readily accessible, provides biomimetic architecture by presenting interwoven fibrils, allows for oxygen and nutrient diffusion, enables uniform distribution of cells in 3D, facilitates cellular reorganization, instructs proper cellular contact, assists differentiation of cells, and supports mineralization. Highly porous scaffolds not only prevent mass transport limitations but also promote vascularization, enable cellular re-organization, and result in formation of functional tissue constructs.

[0006] A common way to study mechanisms of bone formation is the use of cells that can deposit minerals. The process of formation of solid mineral by cells is called biomineralization. This process involves complex cellular interactions and the exact mechanisms of biomineralization are unknown.

SUMMARY

[0007] This disclosure describes biomineralization on paper scaffolds. Three-dimensional (3D) scaffolds for bone tissue engineering are described throughout which bone cells grow, differentiate, and produce mineralized matrix. The paper scaffolds are cultured with cells such as osteoblasts to provide biomineralization. In other embodiments, the cell-seeded paper scaffolds can be co-cultured with cells that promote vascularized bone tissue.

[0008] In one aspect, a method of bone mineralization includes providing a porous three dimensional paper scaffold comprising one or more folded cellulosic paper sheets; seeding

the porous three dimensional paper scaffold with cells capable of producing an inorganic material; and culturing the cell-seeded porous three dimensional scaffold under conditions to induce formation of the inorganic material.

[0009] In one or more embodiments, the porous three dimensional paper scaffold has a honeycomb structure, or a pie shape structure, or a cubic structure, or the porous three dimensional paper scaffold is made of nested concentric cylinders.

[0010] In one or more embodiments, the porous three dimensional paper scaffold includes multiple adjacent compartments having triangular, quadrangular, pentagonal or hexagonal shaped cross section.

[0011] In one or more embodiments, the porous three dimensional paper scaffold has a gradient structure.

[0012] In one or more embodiments, the porous three dimensional paper scaffold includes multiple adjacent compartments having random cross sectional shape.

[0013] In one or more embodiments, the seeding of cells includes introducing a cell-containing hydrogel matrix into the porous three dimensional paper scaffold, and for example, the cells include osteoblast and osteocyte behaving cells.

[0014] In another aspect, a biomineralized scaffold includes a porous three dimensional paper scaffold comprises an outer cellulosic paper surface that supports an inner scaffold structure, the inner scaffold structure comprised of cellulosic paper folded to provide a plurality of multiple adjacent compartments, wherein at least 10 vol% of the scaffold is comprised of an inorganic material.

[0015] In one or more embodiments, the inorganic material comprises a calcium phosphate or the inorganic material is hydroxyapatite.

[0016] In any preceding embodiment, the inorganic material mimics a bone mineral deposit.

[0017] In any preceding embodiment, the mineralized scaffold is made up of at least 20 vol%, at least 30 vol%, at least 40 vol% or at least 50 vol% of the scaffold the inorganic material.

[0018] In any preceding embodiment, the inner scaffold structure comprises a honeycomb structure, or the inner scaffold structure comprises a pie shape structure, or the inner scaffold structure comprises a cuboid structure, or the inner scaffold structure comprises nested concentric cylinders, the inner scaffold structure comprises close packed cylinders.

[0019] In any preceding embodiment, the scaffold further comprises a hydrogel.

[0020] In any preceding embodiment, the scaffold comprises collagen.

[0021] In any preceding embodiment, wherein the scaffold further includes a bone growth promoting material, a vascularization promoting material or an antibiotic.

[0022] The fabrication of partially mineralized scaffolds in 3D shapes is accomplished using paper by folding, and by supporting deposition of calcium phosphate by osteoblasts cultured in these scaffolds. This process generates centimeter-scale free-standing structures composed of paper supporting regions of calcium phosphate deposited by osteoblasts. In one or more embodiments, paper is used as a scaffold to induce template-guided mineralization by osteoblasts. Because paper has a porous structure, it allows transport of O₂ and nutrients across its entire thickness. Paper supports a uniform distribution of cells upon seeding in hydrogel matrices, and allows growth, remodeling, and proliferation of cells. Scaffolds made of paper make it possible to construct 3D tissue models easily by tuning material properties such as thickness, porosity, and density of chemical functional groups. Paper offers a new approach to the study of mechanisms of biomineralization, and perhaps ultimately new techniques to guide or accelerate the repair of bone.

[0023] These and other aspects and embodiments of the disclosure are illustrated and described below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] The invention is described with reference to the following figures, which are presented for the purpose of illustration only and are not intended to be limiting.

[0025] In the Drawings:

[0026] **Figure 1A** is a schematic illustration showing a biomineralization process on a 3D paper scaffold according to one or more embodiments.

[0027] **Figure 1B** is a photograph of a biomineralized scaffold obtained by seeding osteoblasts in the paper scaffold and culturing for 21 days, (i) imaged by a single-lens reflex (SLR) camera (Nikon D5100) noting that the construct is mineralized to an extent that is visible to the naked eye; and (ii)-(iv) imaged by micro-computed tomography (micro-CT) X-Ray scans to illustrate the mineralized areas in the paper constructs in a side view (ii), close up view (iii), and top view (iv); the bright white-colored regions in the micro-CT images demonstrate the mineralized areas in the paper scaffolds.

[0028] **Figures 2A-2B** demonstrate deposition of bone mineral (calcium phosphate) by osteoblasts using Alizarin Red staining, in which **Figure 2A** shows microscope images acquired for biomineralized samples with initial cell densities of 0.1×10^6 , 0.4×10^6 , and 1.6×10^6 cells per sample at days 0, 3, 7, 14 and 21 days; the red color indicates the complex formed by Ca^{2+} ions and Alizarin Red dye; and **Figure 2B** is a bar plot showing an increasing trend in the formation of biomineralized structures as a function of time over 21 days of culture period.

[0029] **Figures 3A-3B** illustrate the amount of hydroxyapatite that was determined using an OsteoImage assay (Lonza, Walkersville, MD). The fluorescent staining reagent (green) binds to the hydroxyapatite portion of the mineralized matrix, and the fluorescence is

measured at 495/519 nm (Ex/Em). The initial cell density was 1.6×10^6 cells/sample. a) The images exhibited green fluorescence proportional to the amount of hydroxyapatite in the samples. b) A progressive increase in the deposition of hydroxyapatite occurred in the paper scaffolds over time.

[0030] **Figure 4A** demonstrates the high-resolution imaging for the minerals that were deposited on paper. The SEM micrographs at different magnifications indicated that mineralized structures formed on the paper constructs after 21 days in culture. The initial seeding density of the cells was 1.6×10^6 cells/sample.

[0031] **Figure 4B** demonstrates the results from the energy dispersive X-ray spectroscopy (EDAX), which illustrated the presence of calcium and phosphate in the mineralized paper scaffolds. EDAX analysis confirmed the elemental presence of phosphorus and calcium on the mineralized paper. We determined a Ca:P ratio of 1.5 ± 0.1 from the results of EDAX analysis.

[0032] **Figures 5A-5B** demonstrates the expression of a bone-specific marker, osteocalcin, which was determined by immunocytochemistry in the paper scaffolds. The initial cell density was 1.6×10^6 cells/sample. We carried out immunostaining for osteocalcin (red) on days 0, 3, 7, 14, and 21, and acquired the fluorescent images by confocal microscopy. We counter-stained the cells with DAPI (blue) to visualize the nuclei of the cells. The expression of osteocalcin increased until day 14 and then decreased. This result could be due to increasing mineralization after day 14.

[0033] **Figures 6A-6B** illustrates the proliferation of cells in the collagen matrix in paper scaffolds at different time points. We stained the nuclei of the cells to image the distribution and proliferation of the cells on days 0, 3, 7, 14, and 21. The initial seeding density was 1.6×10^6 cells/sample. We stained the samples with DAPI (blue), and obtained the images by confocal microscopy. The results indicated that proliferation increased until day 3 and then

decreased after day 7. Because proliferation slows down at the onset of mineralization, this result is expected.

[0034] **Figures 7A-7E** are photographic illustrations of the origami-inspired paper scaffolds for use a biomineralization scaffolds according to one or more embodiments. A) Paper can easily be cut, creased, and folded to form 3D free-standing constructs. B) The paper scaffolds were fabricated to demonstrate different shape, size, and configuration. C) The paper scaffolds can be shaped into 3D structures including gradient patterns. D) We generated a range of different shapes (e.g., triangle, square, pentagon) in the paper scaffolds. E) We also constructed structures using circular features.

DETAILED DESCRIPTION

[0035] Bone is a dynamic structure that continuously remodels throughout its lifetime. Remodeling takes place in response to changes in biomechanical forces, mechanical injury, or to adapt the strength of the bone. An injury to the bone initiates a cascade of complex processes that regenerate the damaged areas. The process of spontaneous healing begins with the formation of a hematoma (blood clot), and elicits an inflammatory response. The hematoma attracts immune cells via signaling molecules. Fibroblasts subsequently migrate towards the site of the injury and lay down extracellular matrix (ECM), which is primarily composed of collagenous proteins (mainly collagen type I) and proteoglycans. Deposition of matrix leads to the formation of a fibrous cartilage (callus, rich in collagen type I), which stabilizes the healing tissue mechanically. As the repair progresses, the callus progressively vascularizes and mineralizes into woven bone, which is eventually replaced by compact bone.

[0036] Bone is composed—in addition to its solid hydroxyapatite structural elements—of four types of cells: osteoclasts, bone-lining cells (also known as osteoprogenitor cells), osteoblasts, and osteocytes. Osteoclasts are multinucleated cells that

are derived from macrophages. The primary function of osteoclasts is to digest bone by secreting acidic proteins and enzymes. The bone-lining cells are of mesenchymal origin from the bone marrow and remain quiescent unless there is an external stimulus (mechanical, hormonal, and/or nutritional). The bone-lining cells turn into osteoblasts when the signaling molecules direct them to deposit bone minerals in response to an external factor (mechanical stimulation, microdamage, and/or injury). Osteoblasts are responsible for formation of bone by laying down collagenous matrix, which is subsequently mineralized by precipitation of calcium and phosphate. During the process of mineralization, some of the osteoblasts are trapped and become buried inside the matrix; there, they terminally differentiate into osteocytes. The osteocytes provide a structural network for the bone.

[0037] In one aspect, a cell platform is described that is capable of biomineralization. Biomineralization refers to a process where an inorganic substance precipitates in an organic matrix. In one or more embodiments, the inorganic substance is a calcium phosphate. In one or more embodiments, the inorganic substance is hydroxyapatite. In one embodiment, osteoblasts are cultured and their deposition of minerals onto cellulose-based structured scaffolds is described.

[0038] In one or more embodiments, the scaffold is a cellulose-based sheet. Cellulose, a polysaccharide with natural origin, has been used for various biomedical applications. Cellulose is the most common organic polymer present on earth, is a biocompatible matter, and does not induce inflammation *in vivo*. Due to its wide availability, it is a well-studied and characterized substance. In one or more embodiments, scaffolds for biomineralization include a substrate made of cellulose. Cellulosic paper has several advantages compared to traditional 3D scaffolds: paper is i) virtually accessible to all laboratories and research centers including resource-poor settings; ii) cost-efficient; iii) highly porous; iv) extremely flexible (can be easily cut, fold, rolled, and manipulated); and v) a biocompatible substrate.

[0039] A cellulosic paper-based cell culture platform for biomineralization of 3D scaffolds is also described. Unlike the traditional strategies, the paper-based cell culture platform for biomineralization is simple and inexpensive and utilizes an extremely flexible substrate. The scaffold includes readily available commercial material, paper that is virtually accessible in any research facility, as the cell culture scaffold. Paper is an ideal material for studying mineralization by bone cells as it naturally possesses highly porous interwoven fibrous architecture, therefore, it enables nutrient and oxygen transport throughout its entire thickness. Paper supports uniform distribution of cells upon seeding in gel matrices and allows for cellular remodeling, proliferation and differentiation of cells in a 3D environment. Furthermore, paper scaffolds can promote vascularization due to their porous nature. Since bone is a vascularized complex tissue, this feature assists in the formation of properly functioning bone and mineralized tissues.

[0040] Paper is a well-characterized, inexpensive, and easily accessible material. It can be conveniently manipulated as a result of its flexible nature. Paper also has highly porous structure and possesses excellent wicking properties. Chemical modification of paper is possible through the -hydroxyl and -carboxyl functional groups of cellulose offering various opportunities to manipulate its chemical nature. In one or more embodiments, biological functional groups can be attached to increase its adhesion properties or render it hydrophobic through silanization or fluorination. In other embodiments, wax-printing (or other patterning techniques) can be used to create hydrophobic boundaries and defined hydrophilic seeding zones on the paper.

[0041] The paper used for the paper scaffold can be any paper that is capable of withstanding sterilization and fabrication operations such as rolling, folding and bending. Exemplary paper includes filter paper, chromatographic paper, card stock and writing stock paper, and the like. The cellulosic fibers contain void and provide a 3D environment in which

the cells can attach and proliferate. Thickness and pore size of the paper are controllable parameters for growing cells in the paper matrix. In one or more embodiment, the pore size of the paper is larger than the diameter of the cells and the thickness is less than 200 μm to prevent diffusion limitations.

For example, Whatman filter paper has a thickness of 190 μm , and therefore, does not block oxygen and nutrient transport that are necessary for cell growth. The paper desirably does not include any binders, or other additives that would interfere with cell seeding and proliferation.

[0042] In other embodiments, 3D biomineralized scaffold can be filled with materials that promote bone growth through the device. These include autograft, allograft, or xenograft bone, bone marrow, demineralized bone (DBM), natural or synthetic bone morphogenic proteins (BMP's i.e. BMP 1 through 7), bone morphogenic-like proteins (i.e. growth and differentiation factor 5 (GFD-5) also known as cartilage-derived morphogenic factor 1, GFD-7 and GFD-8) epidermal growth factor (EGF), fibroblast growth factor (FGF i.e. FGF 1 through 9), platelet derived growth factor (PDGF), insulin like growth factor (i.e. IGF-I and IGF-II and optionally IGF binding proteins), transforming growth factors (TGF- β i.e. TGF- β I through III), vascular endothelial growth factor (VEGF) or other osteoinductive or osteoconductive materials known in the art. Bioactive coatings or surface treatments could also be attached to the surface of the device. For example, bioactive peptide sequences (RGD's) could be attached to facilitate protein adsorption and subsequent cell tissue attachment. Antibiotics could also be coated on the surface of the device or delivered by a material within the device.

[0043] A schematic illustration of a biomineralization process on a 3D paper scaffold is given in **Figure 1A**.

[0044] In steps 1 and 2 of **Figure 1A**, a paper sheet is sterilized and formed into a 3D scaffold (the steps can be performed in any order). Decontamination of the paper should be done before the cell seeding step. Conventional methods for sterilizing paper, such as chemical sterilization, e.g. ethanol or ethylene oxide, nitrogen dioxide, or ozone, and radiation sterilization, e.g., UV radiation, can be used. In one embodiment, sterility of the paper scaffolds can be achieved by soaking them into ethanol and drying prior to contacting them with cells.

[0045] In step 2, the paper is fabricated into a 3D scaffold that approximates the desired geometry of the final biomineralized tissue structure. **Figure 1A**, step 2, shows an exploded view of the paper scaffold. The principles of origami paper folding, in which a small number of basic origami folds can be combined in a variety of ways to make intricate designs, can be used to prepare paper scaffolds of any level of size and complexity. In one or more embodiments, the paper scaffolds are prepared from filter paper. The scaffolds can be free-standing structures having centimeter-scale features of clinically relevant sizes. It is possible to prepare paper scaffolds from mm to cm range.

[0046] **Figures 7A-7E** show a number of exemplary 3D scaffold geometries. Paper is an extremely flexible material and can easily be cut, fold, and manipulated to yield in formation of three dimensional (3D) free-standing constructs, as shown in **Figure 7A**. In general, a paper scaffold includes a continuous outer support layer and a finer internal scaffold made up of interior walls and paper cells or compartments that provide high surface area for biological cell attachment. **Figure 7B** illustrates that paper scaffolds are tunable in shape, size, and configuration. **Figure 7C** illustrates that the paper scaffolds can be shaped into origami structures including gradient patterns, that is, scaffolds having a higher or lower surface area by increasing or decreasing, respectively, the number of internal walls or paper cells. **Figure 7D** demonstrates that the bone scaffold can be fabricated in various geometrical

configurations (e.g., triangle, square, pentagon). In other embodiments, the bone-mimetic structures can include circular features, as shown in **Figure 7E**. The outer support layer and the inner scaffold are not required to be constructed of a single sheet of paper. The inner scaffold cells can be made separately and inserted into the outer support. In certain embodiments, the inner scaffold surfaces can be constructed of individual paper sheets, such as is shown in **Figure 7E**. The concentric inner circles of paper are made from separate rolled paper sheets.

[0047] In a third step, the 3D scaffold is seeded with cells in a hydrogel matrix. Cells that can be grown in the paper-based scaffold can be any prokaryotic or eukaryotic cell. In the current set-up cultured osteoblasts are used to promote biomineralization, however, regeneration of vascular bone tissue co-cultures with endothelial cells is contemplated. In addition to endothelial cells, osteoclasts or fibroblasts can also be co-cultured with osteoblasts.

[0048] The paper scaffold can be seeded by applying a cell-containing hydrogel (or hydrogel precursor) to the paper scaffold and allowing the hydrogel (and suspended cells) to be wicked up into the interior 3D network of the cellulosic fibers. **Figure 1A**, step 3, shows a cell-seeded scaffold, including an exploded view of the embedded cells in hydrogel.

[0049] Any known hydrogel can be used in the methods described herein. Hydrogel matrices are described, for example, in U.S. Patent No. 5,906,934; Lin et al., *Advanced Drug Delivery Rev.* 58: 1379-1408 (2006); and Jen et al., *Biotechnology and Bioengineering* 50: 357-364 (2000). Polymers that can form ionic or covalently crosslinked hydrogels that are malleable can be used in the methods described herein. A “hydrogel”, as used herein, is a substance formed when a polymer is cross-linked via covalent, ionic, or hydrogen bonds to create a three-dimensional open-lattice structure that entraps water molecules to form a gel. The polymer can be an organic polymer. The polymer can be a natural or synthetic polymer.

As used herein, a “hydrogel precursor” is a polymer that can be cross-linked via covalent, ionic, or hydrogen bonds to form a hydrogel.

[0050] Examples of materials that can be used to form a hydrogel include polysaccharides such as alginate, polyphosphazines, and polyacrylates (which are crosslinked ionically) or block copolymers such as Pluronics™ or Tetronics™, polyethylene oxide-polypropylene glycol block copolymers (which are crosslinked by temperature or pH, respectively). Other materials include proteins such as fibrin, polymers such as polyvinylpyrrolidone, hyaluronic acid, and collagen. Collagen is a preferred hydrogel due to its natural presence in bone. Collagen is the most abundant extracellular matrix protein in bone, thus, collagen was used as the gel material in the biomineralization experiments to generate a biomimetic environment. Another reason for using collagen type I is that it is essential in mineralization of bone, therefore, it is physiologically relevant to include it in the cell-encapsulation matrix. In general, these polymers are at least partially soluble in aqueous solutions, such as water, buffered salt solutions, or aqueous alcohol solutions, that have charged side groups, or a monovalent ionic salt thereof.

[0051] Hydrogel precursors include materials that transform into hydrogels under appropriate conditions. Non-limiting examples of polymers with acidic side groups that can be reacted with cations to gel include poly(phosphorene's), poly(acrylic acids), poly(meth acrylic acids), copolymers of acrylic acid and meth acrylic acid, poly(vinyl acetate), sulfonated polymers, such as sulfonated polystyrene. Other no limiting examples include, e.g., alginic acid (AA), carboxymethylcellulose (CMC), ι-carrageenan, poly(galacturonic acid) (PG), poly(acrylic acid) (PAA), and poly(bis(4-carboxyphenoxy)-phosphazene. Copolymers having acidic side groups formed by reaction of acrylic or methacrylic acid and vinyl ether monomers or polymers can also be used. Non-limiting examples of acidic groups

include carboxylic acid groups, sulfonic acid groups, halogenated alcohol groups, phenolic OH groups, and acidic OH groups.

[0052] Non-limiting examples of polymers with basic side groups that can be reacted with anions include poly(vinyl amines), poly(vinyl pyridine), poly(vinyl imidazole), and some imino substituted polyphosphazenes. The ammonium or quaternary salt of the polymers can also be formed from the backbone nitrogens or pendant imino groups. Non-limiting examples of basic side groups include amino and imino groups.

[0053] As used herein, a “gelling agent” is any agent that cross-links a hydrogel precursor to form a hydrogel. For example, a gelling agent can cross-link the hydrogel precursor via covalent, ionic, or hydrogen bonds to form a hydrogel. For example, a water soluble polymer with charged side groups can be ionically crosslinked by reacting the polymer with an aqueous solution containing a gelling agent of opposite charge, e.g., a multivalent ion of the opposite charge. In some embodiments, the polymer has acidic side groups and the gelling agent is a multivalent cation. In other embodiments, the polymer has basic side groups and the gelling agent is a multivalent anion. In some embodiments, the gelling agent is a cation, e.g., Pb^{2+} , Ba^{2+} , Fe^{3+} , Al^{3+} , Cu^{2+} , Cd^{2+} , Ho^{3+} , Ca^{2+} , Zn^{2+} , Co^{2+} , Ni^{2+} , Mn^{2+} , or Mg^{2+} , Sr^{2+} , Gd^{3+} , Pb^{2+} , Ra^{2+} , Fe^{2+} , Pd^{2+} , Bi^{3+} , Hg^{2+} , Au^{3+} , Co^{2+} , Co^{3+} , Cr^{2+} , Cr^{3+} , Mn^{4+} , Pt^{2+} , Pt^{4+} , Sn^{2+} , Sn^{4+} , Ce^{3+} , Ce^{4+} , Ga^{3+} , V^{3+} , or Rh^{3+} .

[0054] In other embodiments, the hydrogel is a temperature-sensitive hydrogel (such as Matrigel or collagen), and gelation is induced by raising the temperature of the substrate to an appropriate level (such as 37 °C). The temperature can be maintained by immersing the substrate within a solution at the appropriate temperature, e.g., in a culture medium suitable for a particular type of cell. Temperature-sensitive hydrogels are known in the art and available commercially. In one embodiment, osteoblasts can be grown in α -minimal essential medium (α -MEM) with 5% fetal bovine serum (FBS) and 5% fetal calf serum (FCS). Upon

seeding the osteoblasts on the paper scaffolds, the culture medium can be changed with the differentiation medium, which contains α -MEM supplemented with 10% (v/v) FBS, ascorbic acid (0.1 mg/mL), and glycerol-2-phosphate (1 mM). The medium can be replaced, for example, every other day during the culture period.

[0055] Once the gel matrix was solidified (for example, for collagen by warming to 37 °C), cells are maintained in position within the thin paper slab of the scaffold. Cells can be grown and maintained at an appropriate temperature and gas mixture (typically, 37 °C, 5% CO₂ for mammalian cells) in a cell incubator, as shown in **Figure 1A**, step 4. Cell-encapsulated paper constructs can be cultured in standard conditions for various time periods, e.g., 0, 3, 7, 14, 21 days or more. In one or more embodiments, different types of cells will require their own media. So the media can be selected from those known to work for that particular cell type.

[0056] The cells deposit minerals, e.g., calcium phosphate, over time over the paper scaffold, producing an inorganic skeleton mimicking the scaffold shape. Figure 1A, step 5, provides an image of a biomineralized structure. In one or more embodiments, at least 10 vol%, at least 20 vol%, at least 30 vol%, at least 40 vol% or at least 50 vol% of the scaffold is mineralized. In other embodiments, scaffold is mineralized between 1-50 vol%, or between 1-40 vol% or between 15-15 vol% or any other range bounded by any value stated herein. Micro-CT analysis shows the deposition of minerals in the origami-inspired paper constructs. Figure 1B shows the hydroxyapatite phase in the mineralized origami-inspired samples. The mineralization of the construct was visible to the naked eye (as imaged by a single-lens reflex Nikon D5100 camera) in Figure 1B, (i). Micro-CT scans then established the distribution of mineralized regions in the paper. The micro-CT images suggest patchy but relatively uniform mineralization (on a scale of 1 cm). Approximately 20% volume of the scaffold was mineralized. Other analyses, such as Alizarin red, phalloidin/DAPI, hydroxyapatite,

osteocalcin staining, and high resolution imaging techniques can be used to confirm the deposition of minerals in the paper constructs.

[0057] This is the first demonstration that biomineralization can take place on a paper medium. The paper-based cell culture platform can be used for the mineralization process by osteoblasts. The samples can easily be evaluated by standard colorimetric assays and microscopic and spectroscopic methods. The ability to shape paper easily into different structures makes it possible to generate specific architectures, and to fabricate individualized scaffolds that can be designed for patient-specific defects.

[0058] In one or more embodiments, the 3D biomineralization scaffold can be used to evaluate the effects of gradients of chemicals and small molecules (e.g. BMP-2, BMP-4, dexamethasone, ascorbic acid) on differentiation into bone lineage.

[0059] In one or more embodiments, the 3D biomineralization scaffold can be used to evaluate the influence of electrical stimulation on the rate of biomineralization or the effects of oxygen tension on biomineralization.

[0060] In another embodiment, the 3D biomineralization scaffold can be used to investigate the effects of mechanical loading on biomineralization.

[0061] In other embodiments, the 3D paper scaffolds can be stacked to form complex 3D structures of paper scaffolds.

[0062] While paper lends itself readily folding, creasing or rolling into three-dimensional structures, multiple layers of the cell-laden paper constructs can be stacked and cultured with mineralizing cells.

[0063] The invention is further illustrated in the following examples, which are presented for the purpose of illustration only and are not intended to be limiting of the invention.

Examples

[0064] Origami-inspired scaffolds were prepared from paper, seeded with mouse osteoblasts, and the osteoblasts were allowed to mineralize these scaffolds. These experiments demonstrated partial mineralization of the paper, and confirm a new strategy for guiding mineralization. The process is described with unfolded paper, however, in application, the paper scaffold can be 3D structures containing 3D folded paper constructs. Unfolded paper structures were used because they were easier to handle than folded structures, and required fewer cells to carry out staining and microscopy analyses (alizarin red, hydroxyapatite staining, osteocalcin staining, SEM).

Materials

[0065] Whatman 114 filter paper, beta-glycerol phosphate, ascorbic acid, alizarin red S and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St Louis, MO). Paraformaldehyde solution (16% (v/v)) was obtained from Electron Microscopy Sciences (Hatfield, PA). Mouse antibodies to Osteocalcin and Alexa 647-labeled antibodies to rabbit IgG were supplied by Abcam (Cambridge, MA). Trypsin-EDTA, penicillin-streptomycin, Calcein AM, Phalloidin (Texas Red-X), fetal calf serum (FCS), fetal bovine serum (FBS), Alpha-minimal essential medium (α -MEM) medium, and Dulbecco's phosphate buffered saline (DPBS) were obtained from Invitrogen (Carlsbad, CA). OsteoImage kit was bought from Lonza Walkersville Inc. (Walkersville, MD). All reagents were used as received without further purification.

[0066] A collagen concentration of 2.5 mg/mL was used as the extracellular matrix material to embed the cells. Higher concentration of collagen causes premature gelation whereas lower concentration is mechanically weak to hold itself within the porous paper matrix.

Cell cultures

[0067] The MLO-A5 cells were suspended in a collagen matrix at different cell densities (0.1×10^6 , 0.4×10^6 , and 1.6×10^6 cells per sample) and 2 μL of the cell-laden gel solution was deposited into the cell-seeding zones of a wax printed 190 μm thick scaffold from Whatman filter paper. Samples were analyzed at different time points to evaluate the progress of the biomineralization process.

[0068] Osteoblasts were grown in α -minimal essential medium (α -MEM) that contained 5% fetal bovine serum (FBS) and 5% fetal calf serum (FCS). FBS and FCS were used to maintain proliferation and differentiation, respectively. The cell cultures were maintained in a standard 37°C incubator equipped to provide 5% CO_2 . Upon preparation of the cell-laden paper scaffolds, the culture medium was changed to differentiation medium, which was simply α -MEM supplemented with 10% (v/v) FBS, ascorbic acid, and beta-glycerol. The medium was changed every 2-3 days to provide a fresh environment for cells

[0069] MLA-O5 cell line represents late osteoblast and early osteocyte behavior. The resulting mineralized matrix from this cell line demonstrates a ratio of calcium to phosphorus similar to that of the native bone. Unlike stem cells or primary cell lines, MLO-A5 cells contain homogeneous population of cells and do not demonstrate variations.

Design and fabrication of the paper-based cell culture platform

[0070] We prepared the free-standing scaffolds using Whatman 114 filter paper. We used these scaffolds to induce template-guided mineralization. For practical reasons, we used flat sheets of paper to carry out the biological assays and microscopy analyses. Wax printing was accomplished with a Xerox Phaser 8560 printer (Xerox Corporation, Norwalk, CT) to create hydrophobic boundaries and spatially defined hydrophilic seeding zones. The paper scaffolds allow 10 samples to be cultured in parallel, and enable the growth of multiple replicates. We ensured the sterility of the paper scaffolds by soaking them in 90% (v/v) ethanol and drying them prior to seeding cells.

[0071] The cell-laden paper constructs were cultured in a standard cell culture incubator up to 3 weeks. Samples were collected at days 0, 3, 7, 14, 21. The samples were analyzed by the following assays to determine whether biomineralization took place in the paper constructs.

Characterization of the mineral deposited in paper scaffolds

[0072] Deposition of the bone mineral (calcium phosphate) by osteoblasts (at initial seeding densities of 0.1×10^6 , 0.4×10^6 , and 1.6×10^6 cells per sample) was assessed by Alizarin Red staining. Alizarin Red selectively binds to calcium and is used to detect mineralization. The samples were stained and visualized by a microscope equipped with a color camera (**Figure 2A**). The red color in the images indicates the complex formed by calcium ions and the Alizarin Red dye. The intensity of red color was quantified using NIH ImageJ Software and the quantified results were converted to bar blots as provided in **Figure 2B**. An increase in the formation of biomineralized structures was observed as a function of time over 21 days of culture period.

[0073] When the initial seeding density was low (0.1×10^6 cells/sample), detectable mineralization did not occur until day 14. The samples with moderate cell density (0.4×10^6 cells/sample) began mineralizing at day 7, whereas the samples with high cell density (1.6×10^6 cells/sample) started mineralizing at day 3. Mineralization began after proliferation of osteoblasts and lay-down of extracellular matrix (primarily collagen). Previous reports suggested that mineralization accelerated as proliferation of cells slowed at higher cell densities.

[0074] Osteoblasts can mineralize effectively when they proliferate and produce sufficient matrix mainly collagen. Mineralization was found to be enhanced when proliferation was decreased in earlier reports. In line of these findings, once the cells with low and moderate initial concentration reached a high number, the deposition of minerals became

similar. At these (low and moderate) initial seeding densities, cells have sufficient room to proliferate until they have no more space to multiply. At this point proliferation slows down and progression of other events such as differentiation and mineralization increase. Initial cell seeding density, regardless of low or high, does not change the final outcome of biomineralization by the end of 3 weeks as after 14 days all samples reach similar cell numbers as a result of proliferation. When cells reach a high concentration, they behave similarly resulting in comparable outcomes in deposition of minerals.

Proliferation of Cells

[0075] We cultured mouse osteoblasts on flat sheets of paper to determine the proliferative activity of the cells on days 0, 3, 7, 14, and 21 (initial cell density: 1.6×10^6 cells/sample). We stained the nuclei of the cells with DAPI (blue) and imaged them by confocal microscopy. The quantified results of fluorescent staining indicated that proliferation increased until day 3 and then slowed (**Figure 6A-B**). Because the cells grow to confluence first, and then differentiate, a decrease in proliferation is expected to correlate with the beginning of mineralization. The actively proliferating osteoblasts deposit ECM (primarily collagen), which is then progressively mineralized. The correlation between proliferation and deposition of minerals has been described in the literature.

Hydroxyapatite content

[0076] We assessed the relative amount of hydroxyapatite (calcium phosphate) in the paper-supported cellular composites with a commercial mineralization kit (OsteoImage, Lonza) by measuring the fluorescence at excitation/emission wavelengths of 495/519 nm (**Figure 3A-3B**). This assay, as described by the manufacturer, uses a fluorescent staining reagent that binds specifically to the hydroxyapatite portion of the biomineralized structures. The intensity of the green fluorescence is proportional to the amount of hydroxyapatite in the sample. The initial cell seeding density was 1.6×10^6 cells/sample on the paper scaffolds.

There was no signal for mineralization at the beginning of the culture period (on day 0) as expected. **Figure 3A-3B** exhibits a progressive increase in the amount of hydroxyapatite in the paper scaffolds as a function of time.

Expression of bone-specific marker on the cells

[0077] We evaluated the expression of bone-specific marker, osteocalcin—a structural matrix protein, (red) by immunocytochemistry. The initial concentration of cells was 1.6×10^6 cells/sample; **Figure 5A** shows fluorescent images obtained by confocal microscopy. The cells were counter-stained with DAPI (blue) to visualize their nuclei. The expression of the bone-specific protein increased until day 14 during mineralization, and decreased afterwards (**Figure 5B**). We attribute this result to increasing mineralization, and consequent transformation of osteoblasts to osteocytes after day 14. It was previously reported that the decrease in expression of osteocalcin after mineralization is due to the entrapment of the cells inside the mineralized matrix. Our results are consistent with this suggestion.

High resolution imaging for deposition of minerals

[0078] We used SEM to image aggregates of hydroxyapatite and cells on paper. After culturing for 21 days, we fixed, dried, and sputter-coated the samples with palladium/platinum (80:20) prior to imaging at 10 kV (**Figure 4A**). We interpret the spherical objects in the SEM micrographs to be particles of hydroxyapatite. Their appearance is similar to those reported in the literature.

[0079] Energy dispersive X-ray spectroscopy (EDAX) analysis established the presence of hydroxyapatite (calcium and phosphate) in the mineralized paper scaffolds (**Figure 4B**). EDAX provided the content of calcium and phosphorus in the samples in weight per cent (%). We then calculated the ratio of calcium to phosphate (Ca:P). The mineral composition was similar to that of the hydroxyapatite, with a Ca:P ratio of 1.5 ± 0.1 (n=7). This ratio is consistent with physiologic deposition of hydroxyapatite by osteoblasts.

Micro-CT analysis

[0080] Micro-CT identified the hydroxyapatite phase in the mineralized origami-inspired samples. Micro-CT provides a 3D image of the sample, and is routinely used to map the deposition of hydroxyapatite. After the growth and culture of the osteoblasts for three weeks, we fixed, dried, and photographed the mineralized paper (**Figure 1B**). The mineralization of the construct was visible to the naked eye (as imaged by a single-lens reflex Nikon D5100 camera). Micro-CT scans then established the distribution of mineralized regions in the paper. The micro-CT images suggest patchy but relatively uniform mineralization (on a scale of 1 cm). Approximately 20% volume of the scaffold was mineralized.

Characterization of biomineralized samples

[0081] Alizarin red staining: At each time point, the samples were fixed with 4% paraformaldehyde. A 2% (w/v) solution of alizarin red was freshly prepared and pH was adjusted to 4.2. The samples were incubated with the alizarin red solution for 20 min, washed and imaged with an upright color microscope. The red color from the images were quantified using NIH ImageJ Software.

[0082] Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDAX): The samples were fixed, frozen in liquid nitrogen, and lyophilized. High-resolution imaging was then performed by Zeiss Supra55VP Field Emission SEM (Carl Zeiss Microscopy, LLC, Thornwood, NY) to visualize the presence of calcium and phosphate in the biomineralized paper constructs.

[0083] Micro-CT: The cells were seeded in the paper scaffolds and cultured up to 21 days. Lyophilized samples were prepared and imaged using a Micro-CT x-ray imaging system (Metris X-Tek, UK). The biomineralized areas on paper constructs appeared to demonstrate bright color.

Statistical analysis

[0084] To analyze the experimental data statistical software GraphPad Prism (Version 4.02, La Jolla, CA) was used. The statistical differences between groups were determined by utilizing one-way ANOVA analyses. p -values < 0.05 were considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

[0085] Unless otherwise defined, used or characterized herein, terms that are used herein (including technical and scientific terms) are to be interpreted as having a meaning that is consistent with their accepted meaning in the context of the relevant art and are not to be interpreted in an idealized or overly formal sense unless expressly so defined herein. For example, if a particular composition is referenced, the composition may be substantially, though not perfectly pure, as practical and imperfect realities may apply; e.g., the potential presence of at least trace impurities (e.g., at less than 1 or 2%) can be understood as being within the scope of the description; likewise, if a particular shape is referenced, the shape is intended to include imperfect variations from ideal shapes, e.g., due to manufacturing tolerances. Percentages or concentrations expressed herein can represent either by weight or by volume.

[0086] Although the terms, first, second, third, etc., may be used herein to describe various elements, these elements are not to be limited by these terms. These terms are simply used to distinguish one element from another. Thus, a first element, discussed below, could be termed a second element without departing from the teachings of the exemplary embodiments. Spatially relative terms, such as “above,” “below,” “left,” “right,” “in front,” “behind,” and the like, may be used herein for ease of description to describe the relationship of one element to another element, as illustrated in the figures. It will be understood that the spatially relative terms, as well as the illustrated configurations, are intended to encompass different orientations of the apparatus in use or operation in addition to the orientations described herein and depicted in the figures. For example, if the apparatus in the figures is

turned over, elements described as “below” or “beneath” other elements or features would then be oriented “above” the other elements or features. Thus, the exemplary term, “above,” may encompass both an orientation of above and below. The apparatus may be otherwise oriented (e.g., rotated 90 degrees or at other orientations) and the spatially relative descriptors used herein interpreted accordingly. Further still, in this disclosure, when an element is referred to as being “on,” “connected to,” “coupled to,” “in contact with,” etc., another element, it may be directly on, connected to, coupled to, or in contact with the other element or intervening elements may be present unless otherwise specified.

[0087] The terminology used herein is for the purpose of describing particular embodiments and is not intended to be limiting of exemplary embodiments. As used herein, singular forms, such as “a” and “an,” are intended to include the plural forms as well, unless the context indicates otherwise.

[0088] It will be appreciated that while a particular sequence of steps has been shown and described for purposes of explanation, the sequence may be varied in certain respects, or the steps may be combined, while still obtaining the desired configuration. Additionally, modifications to the disclosed embodiment and the invention as claimed are possible and within the scope of this disclosed invention.

CLAIMS

What is claimed is:

1. A method of bone mineralization, comprising:
providing a porous three dimensional paper scaffold comprising one or more folded cellulosic paper sheets;
seeding the porous three dimensional paper scaffold with cells capable of producing an inorganic material; and
culturing the cell-seeded porous three dimensional scaffold under conditions to induce formation of the inorganic material.
2. The method of claim 1, wherein the inorganic material comprises a calcium phosphate, or the inorganic material is hydroxyapatite.
3. The method of claim 1 or 2, wherein inorganic material comprises a bone mineral deposit.
4. The method of claim 1, wherein the seeding of cells comprises introducing a cell-containing hydrogel matrix into the porous three dimensional paper scaffold.
5. The method of claim 4, wherein the hydrogel matrix comprises collagen.
6. The method of claim 1, wherein the cells comprise osteoblasts.
7. The method of claim 1, wherein the porous three dimensional paper scaffold comprises and outer surface that supports an inner scaffold structure.
8. The method of claim 7, wherein the inner scaffold structure comprises a honeycomb structure.
9. The method of claim 7, wherein the inner scaffold structure comprises a pie shape structure.
10. The method of claim 7, wherein the inner scaffold structure comprises a cuboid structure.

11. The method of claim 7, wherein the inner scaffold structure comprises nested concentric cylinders.
12. The method of claim 7, wherein the inner scaffold structure comprises close packed cylinders.
13. The method of any of claims 7-12, wherein the outer supporting surface is and the inner scaffold structure are comprised of different paper sheets.
14. The method of claim 13, wherein the inner scaffold structure is comprised of a plurality of paper sheets, each folded to provide single scaffold compartments.
15. The method of claim 13, wherein folding comprises rolling, bending and/or creasing the paper.
16. The method of claim 1, wherein the porous three dimensional paper scaffold comprises of multiple adjacent compartments having triangular, quadrangular, pentagonal or hexagonal shaped cross section.
17. The method of claim 1, wherein the porous three dimensional paper scaffold comprises a gradient structure.
18. The method of claim 1, wherein the porous three dimensional paper scaffold comprises of multiple adjacent compartments having random cross sectional shape.
19. The method of any of the preceding claims wherein the cells are cultured under conditions to provide a mineralized scaffold wherein at least 10 vol% of the scaffold is comprised of the inorganic material.
20. The method of claim 19, wherein the cells are cultured under conditions to provide a mineralized scaffold wherein at least 20 vol%, at least 30 vol%, at least 40 vol% or at least 50 vol% of the scaffold is comprised of the inorganic material.
21. A biomineralized scaffold, comprising:

a porous three dimensional paper scaffold comprises an outer cellulosic paper surface that supports an inner scaffold structure, the inner scaffold structure comprised of cellulosic paper folded to provide a plurality of multiple adjacent compartments,

wherein at least 10 vol% of the scaffold is comprised of an inorganic material.

22. The biomineralized scaffold of claim 21, wherein the inorganic material comprises a calcium phosphate or the inorganic material is hydroxyapatite.

23. The biomineralized scaffold of claim 21 or 22, wherein inorganic material mimics a bone mineral deposit.

24. The biomineralized scaffold of claim 21 or 22, wherein mineralized scaffold is comprised of at least 20 vol%, at least 30 vol%, at least 40 vol% or at least 50 vol% of the scaffold the inorganic material.

25. The biomineralized scaffold of claim 21 or 22, wherein the inner scaffold structure comprises a honeycomb structure.

26. The biomineralized scaffold of claim 21 or 22, wherein the inner scaffold structure comprises a pie shape structure.

27. The biomineralized scaffold of claim 21 or 22, wherein the inner scaffold structure comprises a cuboid structure.

28. The biomineralized scaffold of claim 21 or 22, wherein the inner scaffold structure comprises nested concentric cylinders.

29. The biomineralized scaffold of claim 21 or 22, wherein the inner scaffold structure comprises close packed cylinders.

30. The biomineralized scaffold of any of the preceding claims, wherein the scaffold further comprises a hydrogel.

31. The biomineralized scaffold of claim 35, wherein the scaffold comprises collagen.

32. The biomineralized scaffold of claim 21, further comprising a bone growth promoting material, a vascularization promoting material or an antibiotic.

Figure 1A

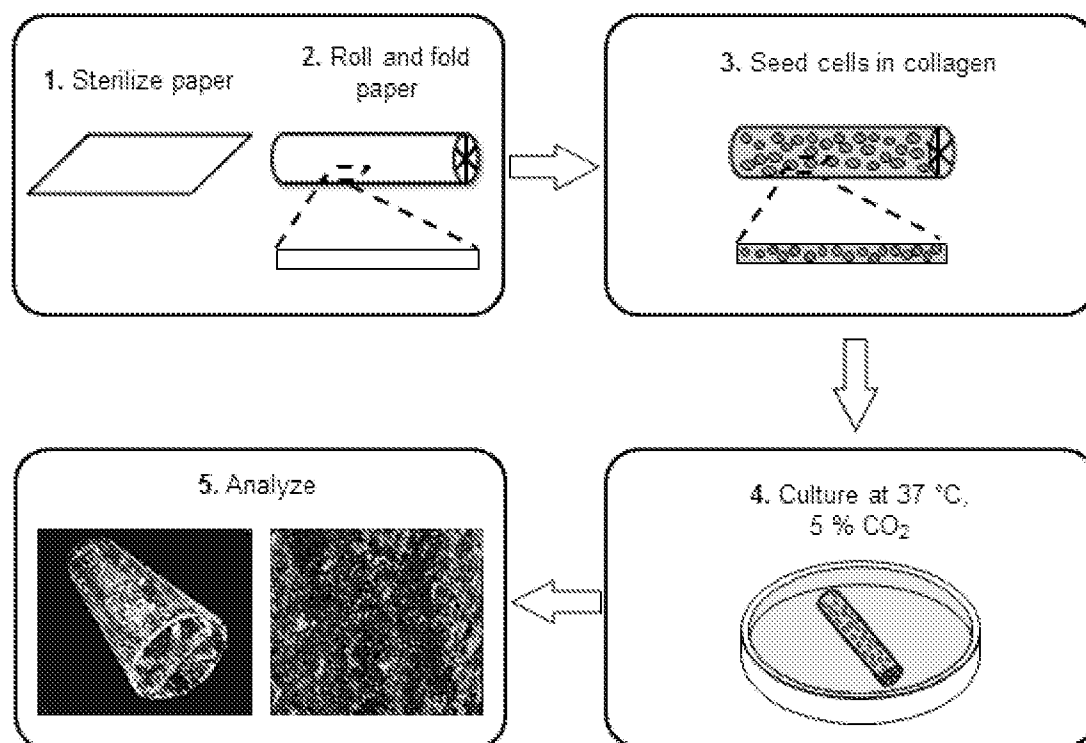


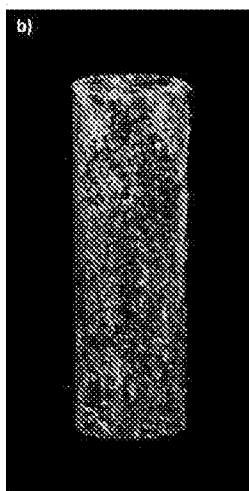
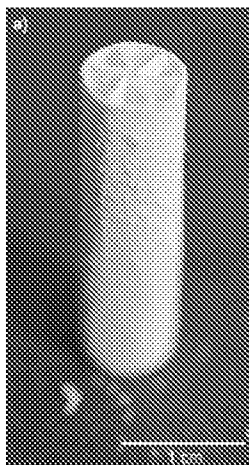
Figure 1B

Figure 2A

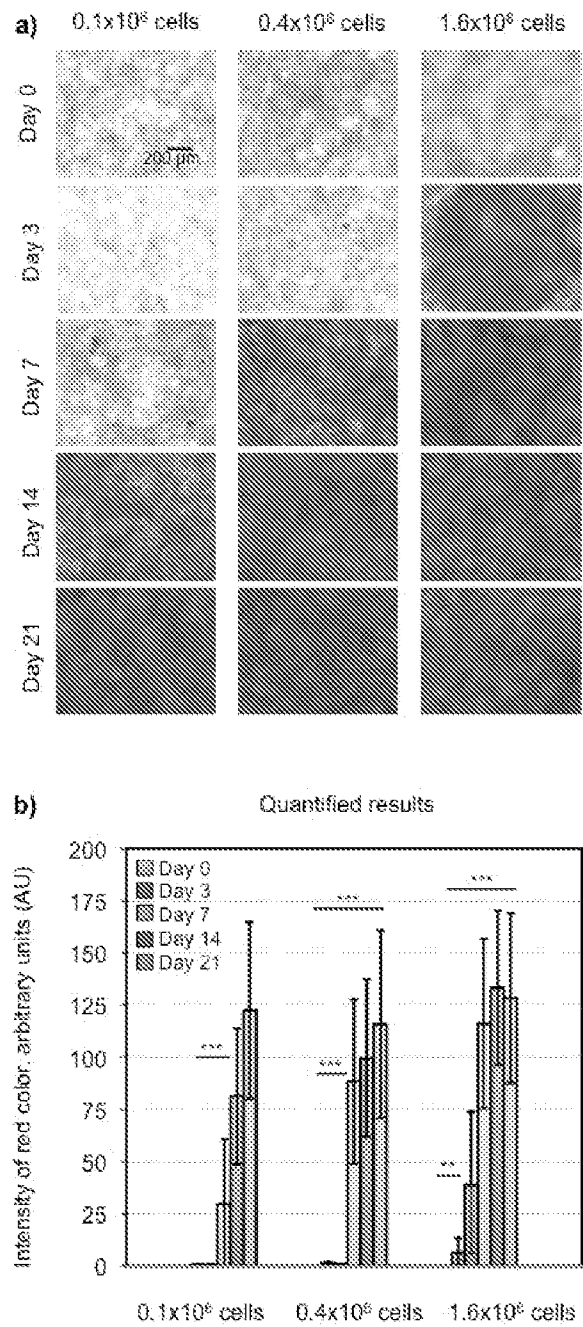


Figure 2B

Figure 3A

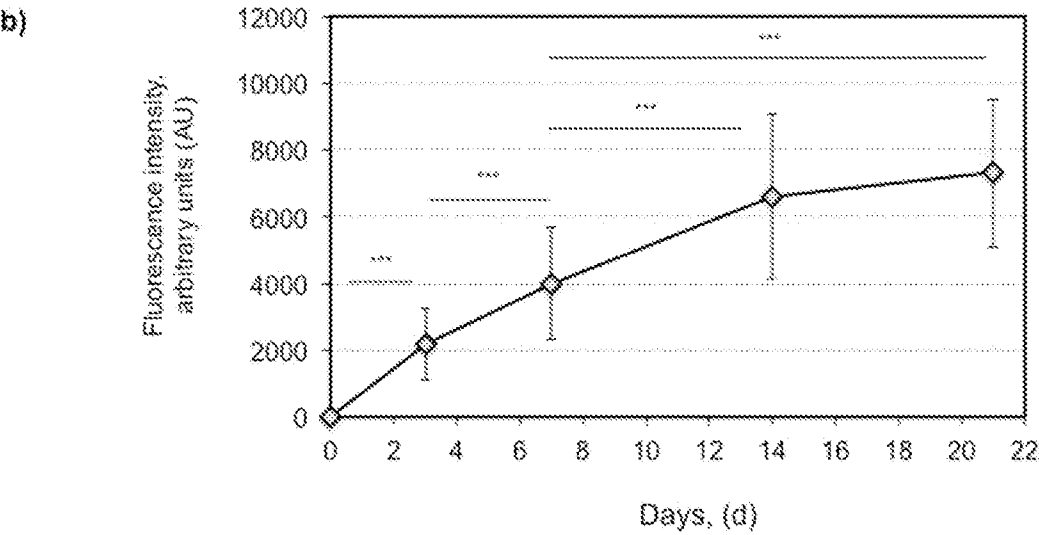
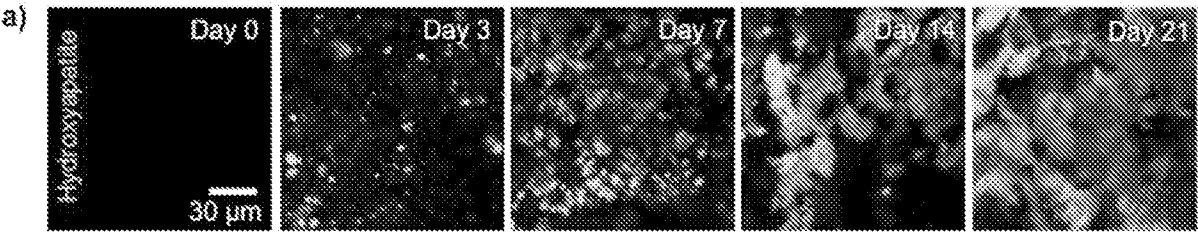


Figure 3B

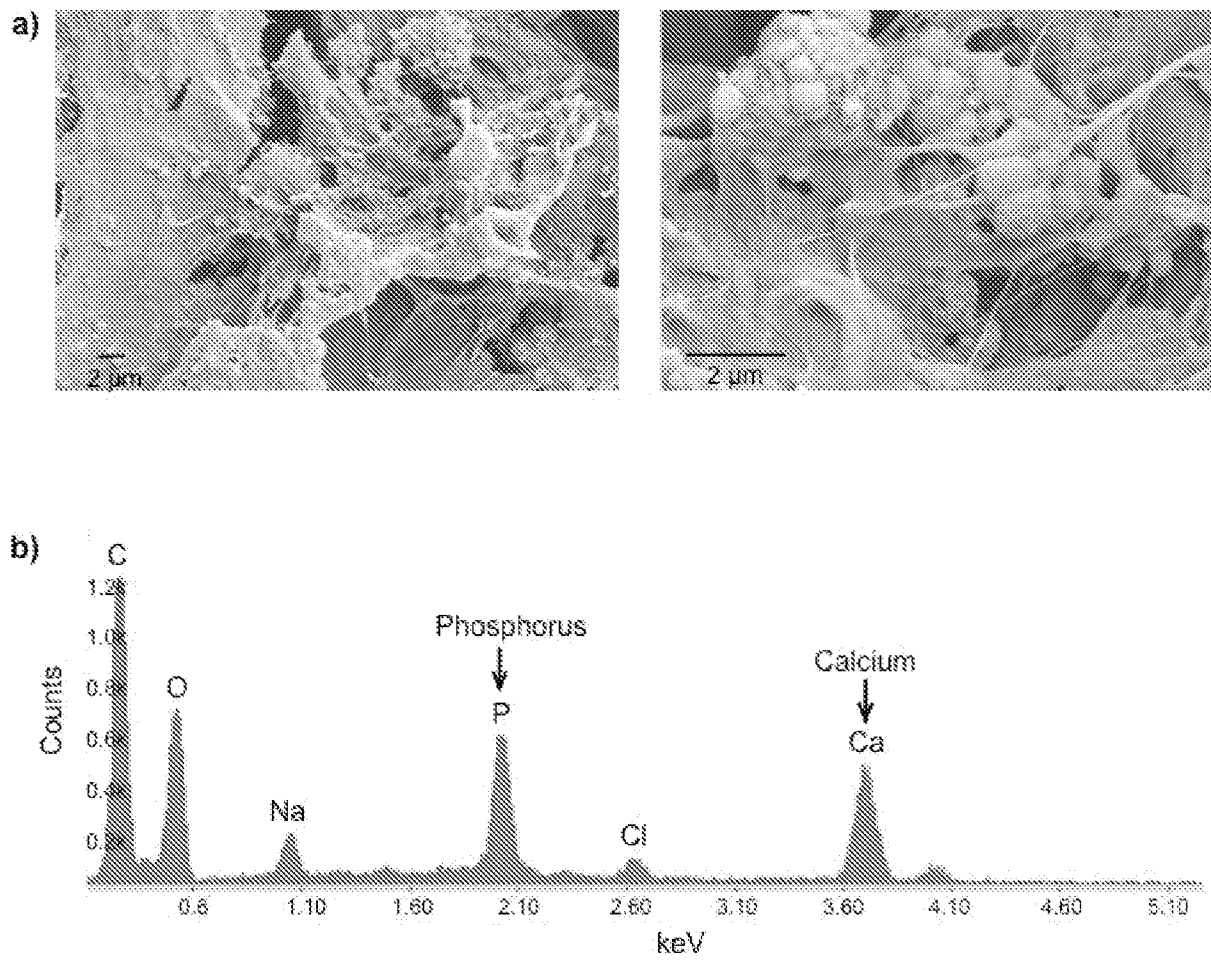
Figure 4A**Figure 4B**

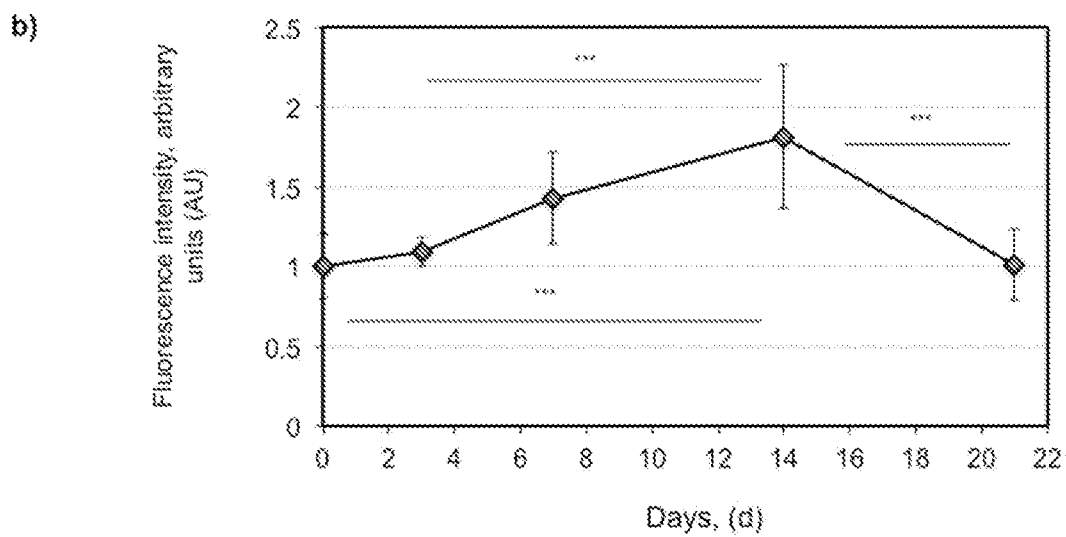
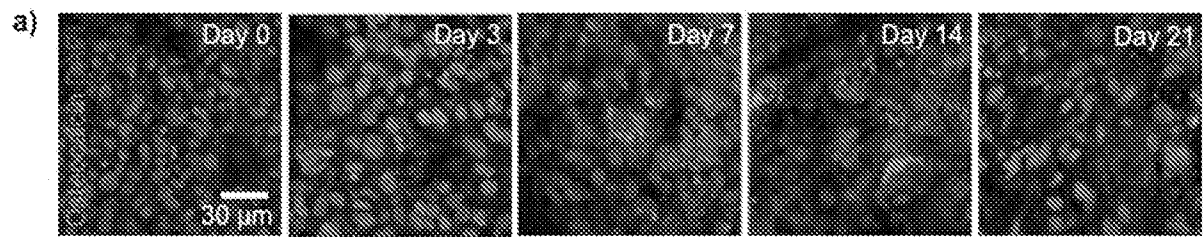
Figure 5A**Figure 5B**

Figure 6A

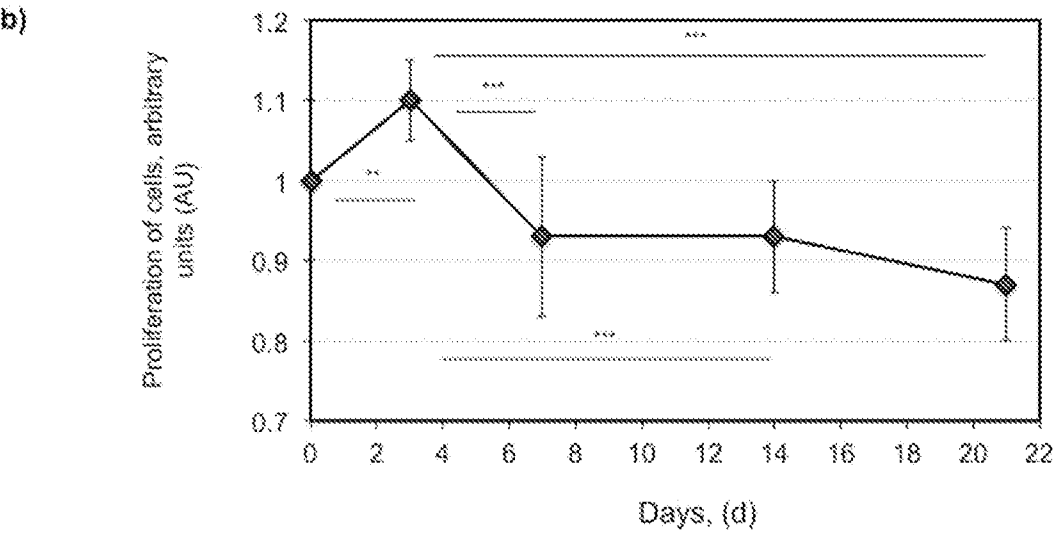
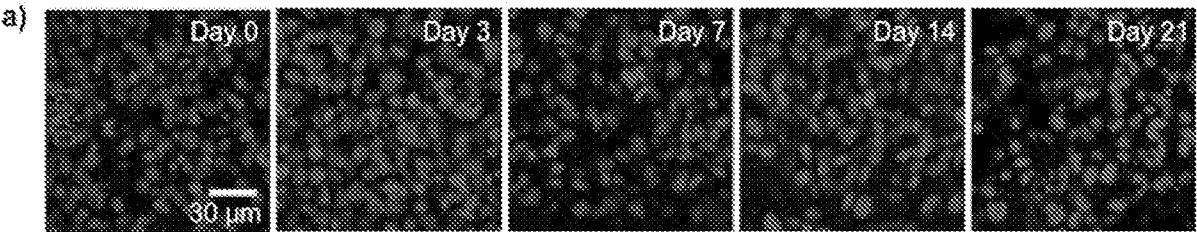
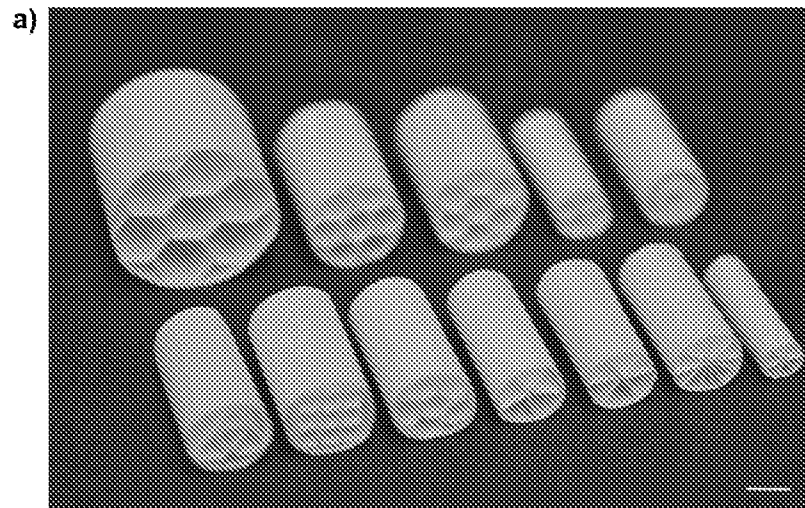
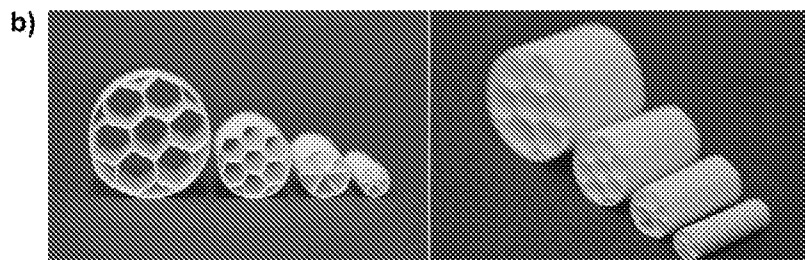
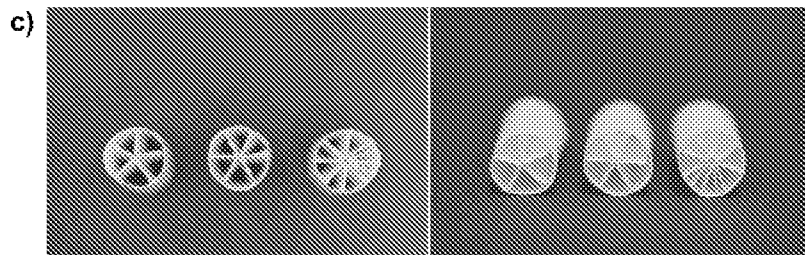
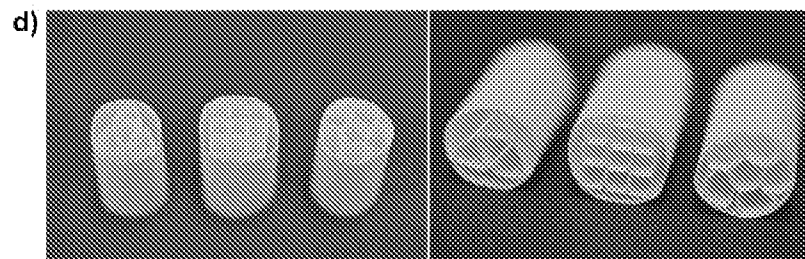
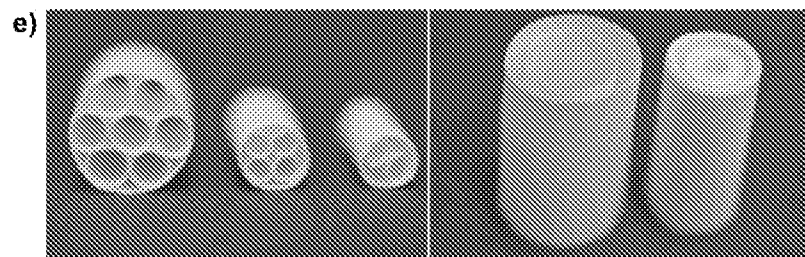


Figure 6B

Figure 7A**Figure 7B****Figure 7C****Figure 7D****Figure 7E**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/16415

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 11/12; A61L 27/24, 27/58; A61F 2/02, 2/28 (2016.01)

CPC - C12N 11/12; A61L 27/24, 27/58; A61F 2/02, 2/28

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)

IPC(8) Classification(s): C12N 11/12; A61L 27/24, 27/58; A61F 2/02, 2/28 (2016.01)

CPC Classification(s): C12N 11/12; A61L 27/24, 27/58; A61F 2/02, 2/28

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)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google Scholar; IP.com; EBSCO Discovery; 3D scaffold, seeded, cultured, osteogenic cells, calcium, collagen hydrogel, osteoblast cells bone mineralization, paper-based scaffold, cell culture, cuboid, honeycomb, nested cylinders

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	(XING, Q et al.) Porous biocompatible three-dimensional scaffolds of cellulose microfiber/gelatin composites for cell culture. Acta biomaterialia. 2010. Vol. 6. No. 6; pages 2133-2135	1-2, 3/1-2, 4-12, 13/7-12, 14/13/7-12, 15/13/7-12, 16-18
Y	(HIGASHI, K et al.) Nematic ordered cellulose templates mediating order-patterned deposition accompanied with synthesis of calcium phosphates. Cellulose. 2012; abstract; page 83	2, 3/1-2
Y	US 2008/0131473 A1 (BROWN, R et al.) 5 June 2008; figure 1, paragraphs [0016], [0026], [0060], [0123], [0180]-[0181], [0191]; claims 1, 22-23, 25	1-2, 3/1-2, 4-8, 11-12, 13/7-12, 14/13/7-12, 15/13/7-12
Y	(ITO H, et al.) A honeycomb collagen carrier for cell culture as a tissue engineering scaffold. Artificial organs. 2001. Vol. 25. No. 3; abstract; figure 1; pages 214-215	8, 12, 13/8, 13/12, 14/13/8, 14/13/12, 15/13/8, 15/13/12
Y	US 2006/0172417 A1 (RATHENOW, J et al.) 3 August 2006; abstract; figure 2b; paragraphs [0043], [0052], [0136], [0153]	1, 7, 9-10, 13/9-10, 14/13/9-10, 15/13/9-10
Y	US 5,622,857 A (GOFFE, RA) 22 April 1997; figure 10a; column 2, lines 44-52; claim 1	11, 13/11, 14/13/11, 15/13/11
Y	WO 2014/159722 A1 (LIVERMORE-CLIFFORD, C et al.) 02 October 2014; paragraphs [0006], [0020], [0029]-[0030], [0034]-[0037], [0047]; figures 1, 4-5	1, 13/7-12, 14/13/7-12, 15/13/7-12, 16-18

☐ 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 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

20 March 2016 (20.03.2016)

Date of mailing of the international search report

03 JUN 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/16415

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.:
because they relate to subject matter not required to be searched by this Authority, namely:

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.: 19-20, 30
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:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-18 are directed toward a method of bone mineralization.

Group II: Claims 21-29 and 31-32 are directed toward a biomaterialized scaffold.

Continued Within the Next Supplemental Box

1. ☐ As all required additional 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 additional fees, this Authority did not invite payment of additional fees.
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.:
1-2, 3/1-2, 4-12, 13/7-12, 14/13/7-12, 15/13/7-12, 16-18

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.

-Continued from Box III: Observations where unity of invention is lacking-

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include a method for seeding the porous three dimensional paper scaffold with cells capable of producing an inorganic material; and culturing the cell-seeded porous three dimensional scaffold under conditions to induce formation of the inorganic material, which are not present in Group II; the special technical features of Group II include a scaffold comprised of an outer cellulosic paper surface that supports an inner scaffold structure, the inner scaffold structure comprised of cellulosic paper folded to provide a plurality of multiple adjacent compartments, wherein at least 10 vol percent of the scaffold is comprised of an inorganic material, which are not present in Group I.

The common technical features of Groups I-II is a porous three dimensional paper scaffold comprising one or more folded cellulosic paper sheets.

This common technical feature is disclosed by US 2006/0172417 A1 to Rathenow, et al. (hereinafter 'Rathenow') in view of US 6,800,753 B2 (Kumar). Rathenow discloses a porous three dimensional paper scaffold (a porous three dimensional paper supporting bodies; figure 1; paragraphs [0017], [0046]-[0047], [0052]) comprising one or more folded paper sheets (three-dimensional folded paper sheet as a supporting body; abstract; figure 2b). Rathenow does not disclose the scaffold comprising cellulose. However, Kumar discloses a scaffold comprising cellulose (scaffold comprising cellulose; claim 1). It would have been obvious to one of ordinary skill in the art, at the time the invention was made, to have modified the method, as previously disclosed by Rathenow, in order to have provided for cellulose, as disclosed by Kumar, so as to obtain scaffolds having optimal biodegradability and flexibility.

Since the common technical feature is previously disclosed by Rathenow in view of Kumar, this common feature is not special and so Groups I-II lack unity.