ABSTRACT

ELECTROSPUN NANO-COMPOSITE SCAFFOLD BASED ON POLY-L-LACTIDE-CO-ε-CAPROLACTONE, GELATIN AND NANO-HYDROXYAPATITE

The present invention is directed towards a bone graft material, formable bone composition for application to a bone defect site to promote new bone growth. In particular, the present invention relates to a bone substitute material which is a nano-composite membranous scaffold of a combination of Poly-L-Lactide-co-ε-caprolactone, (PLC)/gelatin/nano-hydroxyapatite developed by the process of electrospinning

We Claim:

1. A nano-composite scaffold for bone growth comprising: a Poly-L-Lactide-co-ε-caprolactone, (PLC), gelatin, and nanocrystalline hydroxyapatite (n-HA).

2. A nano composite scaffold as clamed in claim 1, wherein the Poly (L-lactide-co-ε-caprolactone, PLC) is made from L-lactide (PLA) and caprolactone (PCL) in a ratio of 70: 30.

3. The nano-composite scaffold as claimed in claim 1, wherein the ratio of PLC to gelatin is 3:1 by weight.

4. A nano composite scaffold as clamed in claim 1, wherein the weight of hydroxyapaptite in the composition is 40% to the total weight of PLC-Gel mixture.

5. A method of producing a scaffold for bone growth according to claim 1 comprising the steps of:
   a) preparation of a polymer solution;
   b) mixing the polymer solution with gelatin in a ratio of 3:1 in a solvent;
   c) mixing a dispersion of hydroxyapatite powder with the PLC-Gel mixture obtained in step b);
   d) electrospinning the PLC-Gel-HA polymer mixture; and
   e) storing said scaffold under vacuum.

6. The method as claimed in claim 5, wherein the electrospinning comprises:
   a) filling a syringe with the polymer solution and loading into a syringe pump;
   b) connecting the syringe needle tip to a positive output of a high voltage supply while the aluminium foil covered collector is grounded; and
   c) passing a voltage of about 12KV and maintaining a flow rate at 1.5mL/h.

7. The method as claimed in claim 5, wherein the polymer solution has a concentration of 12% w/v.

8. The method as claimed in claim 5, wherein the polymer solution is prepared in a solvent selected from 1,1,1,3,3,3-hexafluoro-2-propanol.

9. The method as claimed in claim 5, wherein the weight of hydroxyapaptite used is 40% to the total weight of PCL-Gel mixture.

10. The method as claimed in claim 8, wherein the solvent used for dispersing hydroxyapaptite is 1,1,1,3,3,3-hexafluoro-2-propanol.

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FIELD OF THE INVENTION

The present invention is directed towards a bone graft material, formable bone composition for application to a bone defect site to promote new bone growth. In particular, the present invention relates to a bone substitute material which is a nano-composite membranous scaffold of a combination of Poly-L-Lactide-co-ε-caprolactone, (PLC)/gelatin/nano-hydroxyapatite (PGH) developed by the process of electrospinning. It particularly originates from the bone substitute material disclosed in our co-pending Indian patent Application No. 3594/MUM/2012, covering a nano-composite, membranous scaffold of combinations of Poly(L-lactic acid)/gelatin/nano-hydroxyapatite (PGH).

The desire to produce an improved material for facilitation of bone growth and adjacent tissue regeneration has led to this invention. There are many applications for such a material, including repair of severely wounded osseous defects (including cranial repair and critical fracture repair), skeletal (especially facial) reconstruction procedures, conformal coating at defect site and periodontal surgery aimed in part at regeneration of bone loss from periodontal disease. The developed product presents itself as a cost-effective material to be used in above said applications, without needing expensive drugs or growth factors and toxic cross linking agents.

The flexibility and degradation properties of the scaffolds are very important parameters in bone regeneration. Poly (L-lactide-co-ε-caprolactone, PLC), made of poly lactic acid (PLA) and PCL, is a highly elastic and linear biodegradable polymer which exhibit a relatively milder degradation property than PLA, PGA and PCL polymers. Due to which, it avoids an abrupt drop in pH during degradation unlike PLA, PGA and PCL scaffolds. PLC 7015 polymer is used for suturing wires, bone plates, and bone screws, with tuned degradation times in the human body. Thus, the mild degradation property provides a technical advantage to current electrospun material over the previously reported materials.

BACKGROUND OF THE INVENTION

Bone repair is a subject of intensive investigation in human health care. Scaffolds for bone tissue engineering are often designed to serve as a temporary, artificial extracellular matrix (ECM) in order to support cell attachment and guide three-dimensional (3D) tissue formation. As such, it is desirable for the scaffold to mimic the advantageous characteristics of the natural ECM. Despite advancement in scaffolding technologies, development of scaffold which mimics the structure and function of native bone is a major challenge in bone tissue engineering. The role of scaffold is not only to provide structural support but also to mimic architecture of native extra cellularmatrix (ECM).

Surgical implants should be designed to be biocompatible in order to successfully perform their intended function. The extracellular matrix (ECM) of bone consists of both organic and inorganic phases. The architecture of bone ECM is fibrous with fiber diameters ranging from tens of nanometers to a few micrometers. Electrospun scaffolds mimic the structure of native bone ECM and modulate cell adhesion and enhance osteogenic differentiation during bone regeneration process.

Polymers as a biomaterial has gained considerable interest for various biomedical applications, mainly due to their design flexibility, presence of functional group moieties and biodegradation of certain polymers at body pH. The most commonly used synthetic polymers are poly(lactic-coglycolic) acid (PLGA), poly lactide acid, polycaprolactone (PCL), polyurethane and poly(hydroxybutyrate)-*co*-hydroxyvalerate. Synthetic polymers are gaining popularity as biomedical materials due to their easy scale-up production and also they are mechanically stronger than natural polymers. Properties such as molecular weight, crystallinity, degradation rate can also be controlled depending upon scaffold design. The most commonly used synthetic polymers are poly(lactic-co-glycolic) acid (PLGA), poly lactide acid (PLA), polycaprolactone (PCL), polyurethane and poly(hydroxybutyrate-cohydroxyvalerate.

Among these, PLA and PLGA are the most widely used polymers due to their biocompatibility and non-toxic biodegradability. PLGA is often used in medical applications because its degradation product, glycolic acid, is a natural metabolite and also be excreted out as urine. PLGA has also been used as delivery vehicle for drugs, proteins and DNA.

Various bioactive ceramic scaffolds such as hydroxyapatite, tri-calcium phosphate, bioactive glass, have been tried for bone regeneration in vivo. They have shown excellent biocompatibility and proved to be osteointegrative. But the major limitation of pure ceramic scaffolds in clinical condition is their brittleness and lack of compressibility. They cannot take a shape of irregular contour defect.

Various polymers such as poly(lactide-co-glycolide) (PLGA), poly-(l-lactic acid) (PLLA), polycaprolcatone (PCL) have been blended with ceramics to improve their ductility and mechanical property. Methods such as solvent casting-particulate leaching (SC-PL) and phase separation have been used for preparation of polymer/ceramic composite scaffolds. However, these methods limit the exposure of ceramic particles on the surface of the biomaterial due to embedding of ceramic particles deep inside the dense polymer matrix. Moreover, limited presence of osteoconductive ceramic particles over surface of scaffold decreases the chances of cells to get enough cues for differentiation. Furthermore, agglomeration of HA particles also results into poor mechanical properties of scaffold.

Composites of PCL/HA have been reported in the literature and have been shown to have good mechanical properties and also to induce bone cell growth. However, these composites are generally too stiff to be shaped at room temperature.

In the literature, several patents describe products, which aim to solve the above needs for Example,

WO2007015208A describes an injectable bone scaffold comprising poly (vinyl alcohol), water and tricalcium phosphate, which upon mixing generates a hydrogel. Depending on the

amount of polymer present, the composite can be readily injected in cavities in the time range of 2-60 minutes. The hardening is induced by leakage of water into the surrounding media. However, unlike the invention herein, this patent application employs a polymer, which is degraded very slowly in the human body. Furthermore, the composite comprises a non-porous and dense bone scaffold.

US6331312 describes a method of producing a bone scaffold material, consisting of poorly crystalline apatite together with biodegradable polymers. The product is mixed with water, which creates a mouldable composite. However, unlike the invention herein, this patent application describes preparation routes mainly intended for attaining non-porous and dense composites.

US7004974 describes a substance, which consists of calcium phosphate granules, lipid and hyaluronic acid. When mixed with water, this substance generates a mouldable and injectable composite, with relatively low compression strengths.

US2006013857 describes different compositions, which have the form of putty at body temperature and which are hard at room temperature. The compositions contain gelatin, calcium stearate, tocopheryl acetate and in some examples microsized HA particles (6-12 μm). This document does not describe the use of nanosized HA.

There are a few documents on the use of biodegradable polymers together with HA and plasticizers. US7186759 for example, describes a three component system consisting of a biocompatible polymer, a water-soluble or hydrolytically degrading polymer, such as poly(ethylene glycol) and a bioactive substance. However, even though patent US7186759 discloses a composite that contains a porogen in the form of a water-soluble or hydrolytically degrading polymer, the mouldability has proven to be restricted to a short period of time. It should also be noted that the degradation speed of PEG is in the same range as the degradation of the supporting polymer and the desired porosity of the composite upon

removal of the PEG polymer will not be very efficient. Furthermore, the patent employs micrometer sized HA particles.

WO2008000488 describes a biomaterial for tissue regeneration, which may consist of a bioactive material, such as beta-tricalcium phosphate, a biodegradable polymer, such as poly(lactic-co-glycolic acid), and a water binding agent, such as calcium sulfate, to decrease the degradation of the biodegradable polymer. This document also describes the use of a compound, such as poly(ethylene glycol) 400, to improve the dissolution of the biodegradable polymer. This document does not describe the use of nanosized HA.

Other references which disclose such biomaterial scaffolds include US20060142736, US20100312340, WO2010100277, US20120308510, and US20130310491, but none of the documents disclose the use of a combination of nanosized HA, PLC polymer and gelatin for obtaining a biocompatible, biodegradable, osteoconductive, nanofibrous, membranous scaffold.

Thus, one of the objects of this invention is to provide a biocompatible, biodegradable, osteoconductive, nanofibrous, membranous scaffold that mimics the natural bone, both in composition and microstructure so as to be useful in the manufacture of bone substitute materials circumventing the drawbacks encountered in prior art.

It is also one of the objects of the present invention to provide a cost-effective biomaterial that can be used safely and effectively for repairing bone defects. Most of the materials used in the prior art consist of either sophisticated cell-material construct or material- growth factor construct, which are very much costly. These practices are either complicated or expensive. Hence, the present inventors thought of a bone biomaterial with specific design and architecture that could regenerate new bone effectively and is cost-effective.

The inventors of the present invention have thus endeavored to prepare a nano-composite membranous scaffold that promotes excellent bone repair and regeneration without the drawbacks of the conventional methods.

BRIEF DESCRIPTION OF FIGURES

Figure 1: Scanning Electron Microscopy (SEM) Morphology of electrospun (a) PLC, (b) PLC/Gel, and (c) PLC/Gel/n-HA scaffold.

Figure 2: Transmission Electron Microscopy (TEM) Morphology of electrospun (a) bright field (d) dark field of PLC, (b) bright field (e) dark field of PLC and and (c) bright field (f) dark field PLC/Gel/HA nanofibers scaffold

Figure 3: 3D surface topography of nanofibrous scaffold using atomic force microscopy (a) PLC, (b) PLC/Gel, and (c) PLC/Gel/n-HA scaffold. (Scale- 1 μm).

Figure 4: Bar graph showing % hemolysis for PLC, PLC/Gel and PLLA/Gel/n-HA scaffolds

Figure 5: SEM micrographs of platelet adhesion on (a) PLC, (b) PLC/Gel, and (c) PLC/Gel/n-HA scaffold

Figure 6: SEM micrograph of MG-63 cell lines attached on PLC (a,d), PLC/Gel (b,e) and PLC/Gel/HA (c,f) scaffold after 3,5 days of culture.

Figure 7: CLSM image of attachment of MG-63 cell lines on (a) PLC, (b) PLC/Gel and (c) PLLA/Gel/n-HA scaffolds. The cell nucleus was stained with PI (red) and filament with FITC-phalloidin (green)

SUMMARY OF THE INVENTION

One aspect of the present invention relates to a bone substitute nano-composite scaffold of a combination of Poly-L-Lactide-co-ε-caprolactone, (PLC)/gelatin/nano-hydroxyapatite (PGH). This scaffold material can be used in a variety of compromised bone growth conditions, impaired bone healing and adjacent tissue regeneration procedures.

Another aspect of the present invention relates to a process for preparation of the nano-composite scaffold of the combination of PLC/gelatin/nano-hydroxyapatite comprising the steps of:

a) Preparation of a polymer solution;
b) mixing the polymer solution with gelatin in a ratio of 3:1 in a solvent;
c) mixing a dispersion of hydroxyapatite powder with the PLC-Gel mixture obtained in step b); and
d) electrospinning the PLC-Gel-HA polymer mixture; and
e) storing said scaffold under vacuum.

Further scope and applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating embodiments of the invention, are given by way of illustration only, because various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

DETAILED DESCRIPTION OF THE INVENTION

For the purposes of the following detailed description, it is to be understood that the invention may assume various alternative variations and step sequences, except where expressly

specified to the contrary. Moreover, other than in any operating examples, or where otherwise indicated, all numbers expressing, for example, quantities of ingredients used in the specification are to be understood as being modified in all instances by the term "about". It is noted that, unless otherwise stated, all percentages given in this specification and appended claims refer to percentages by weight of the total composition.

Thus, before describing the present invention in detail, it is to be understood that this invention is not limited to particularly exemplified systems or process parameters that may of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to limit the scope of the invention in any manner.

The use of examples anywhere in this specification including examples of any terms discussed herein is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to various embodiments given in this specification.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In the case of conflict, the present document, including definitions will control.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a "polymer" may include two or more such polymers.

The terms "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

As used herein, the terms "comprising" "including," "having," "containing," "involving," and the like are to be understood to be open-ended, i.e., to mean including but not limited to.

As used herein, the term "Extra cellular matrix" intends to mean an intricate network of macromolecules occupying the spaces between the cells of multicellular animals. It is particularly important in the reinforcement of support tissues. Under the light microscope, it appears as a structureless mass of ground substance with embedded connective tissue fibers. The ground substance consists primarily of proteoglycans and glycoproteins, whereas the fibers consist mainly of fibrous proteins such as collagen.

As used herein, the term "nanostructure" refers to any structure wherein atleast one dimension of the structure is at the sub-micron i.e. nonoscale range.

As used herein, the term "hydroxyapatite" or "HA" refers to a naturally occurring mineral form of calcium apatite with the formula $Ca_5(PO_4)_3(OH)$.

As used herein, the term "scaffold" or "matrix" can be used interchangeably and they relate to a semi-solid system comprising a three-dimensional network of one or more species of polysaccharide chains. Depending on the properties of the polysaccharide (or mixtures of polysaccharides) used, as well as the nature and density of the network, such structures in equilibrium can comprise various amounts of water.

As used herein, the term "nano-hydroxyapatite" or "n-HA" refers to hydroxyapatite particles having a size comprised between 10 and 100nm, preferably 20 and 80nm, preferably 30 and 70nm, preferably 30 and 60nm and more preferably about 50nm.

One aspect of the present invention thus in accordance is related to the development of a new nano-composite material for bone tissue engineering, namely, a biocompatible, biodegradable,

osteoconductive, nanofibrous, membranous nano-composite scaffold of a combination of Poly (Lactide-co-ε-caprolactone, PLC)/gelatin/nano-hydroxyapatite (PGH).

Combinations of nano-hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and biopolymers like Poly-L-Lactide-co-ε-caprolactone, (PLC) provide ideal environments for effective bone regeneration. The spontaneous bone structure is a hybrid structure of the inorganic nano-hydroxyapatite and the organic extracellular matrix (ECM) in which collagens give structural support to resident cells.

Poly-L-Lactide-co-ε-caprolactone, (PLC), made of 70/30 L-lactide (PLA)/caprolactone (PCL) copolymer, is a highly elastic and linear biodegradable polymer with a relatively mild degradation, which avoids an abrupt drop in pH value during degradation unlike polylactic acid (PLA) and polyglycolic acid (PGA) scaffolds. This copolymer comprising of 70 % (by weight) of lactide and 30 % (by weight) of ε-caprolactone is suitable for use in biomedical engineering such as bone reconstructive surgery.

In an embodiment, the ratio of the polymer PLC to gelatin ranges from about 1:3 to 3:1, preferred ranges being 3:1.

The fiber-forming polymer in the scaffold of the present invention is Poly-L-Lactide-co-ε-caprolactone. The fibrous structure of PLC allows guided bone regeneration as it may act as a mechanical barrier to prevent the ingrowth of the un-necessary connective tissue into the defect simultaneously allowing the repopulation surrounding bone cells and thus, promote osteogenesis without interference from other tissue types.

The scaffold is made of porous materials like PLC and gelatin made from biodegradable materials suitable for tissue regeneration. Other non-limitative examples of such biodegradable materials include poly(L-Lactic acid) (PLLA), polyglycolic acid (PGA), poly(lactide-co-glycolide), and/or mixtures thereof. Further degradable natural macromolecules (typically enzymatically degradable) such as gelatin, collagen, other proteins

and their derivatives may also be used. Gelatin is particularly suitable for the porous materials (e.g., when the porous material is a scaffold) in part because gelatin is a denatured biopolymer derived from collagen by acidic or basic hydrolysis. Gelatin generally circumvents risks of immunogenicity and pathogen transmission often associated with acellular bone matrix collagen, while providing similar advantageous biological properties.

Other bioceramics which can be used in place of hydroxyapatite (HA), include carbonate hydroxyapatite, tricalcium phosphate (TCP), carbon, calcium carbonate, and 'Bioglass' (a highly bioactive glass particulate material used in bone repair) and the like.

Among the apatite group, hydroxyapatite (HA) and its various derivatives or variants, have been recognized to be a major structural component of biological tissues (e.g., bone, teeth and some invertebrate skeletons). The presence of HA in a scaffold not only mimics the natural extracellular matrix (ECM) but also enhances the mechanical properties, degradation profile and creates a 3-D microenvironment for better cellular interaction and proliferation.

In addition to the porosity of the bone scaffold, the crystal size of the HA is also important to stimulate the growth of endogenous bone. For certain biomaterial applications, it is highly desirable to use nanosized HA, ie. with a particle size of 1 - 100 nm in length. It is generally considered that the bioactivity of HA is improved if the HA crystals are of a similar size and shape as those produced by the human body. The body recognizes the nanosized HA as a part of its own bone tissue and starts to grow new bone around the foreign object. For implants, a coating with nanosized HA will significantly increase the bone cell activity compared to microsized HA. For polymer/HA composites, the bioactivity as well as the strength is greatly improved with nanosized HA.

"Nano-hydroxyapatite" or "n-HA" refers to hydroxyapatite particles having a size comprised between 10 and 100nm, preferably 20 and 80nm, preferably 30 and 70nm, preferably 30 and 60nm and more preferably about 50nm. Preferably, the n-HA suitable for carrying out the present invention is a n-HA prepared by chemical precipitation at room temperature, for

example by precipitation of a solution of phosphoric acid with a solution of calcium hydroxide.

The n-HA is deposited on the PLC and PLC-GA scaffold by alternate soaking method which forms apatite by incubating the substrate in Ca^{2+} and PO_4^{-3} solutions alternatively for a certain number of cycles.

The nanosized HA is synthesized with a method, which involves the mixing of an aqueous dispersion of a calcium compound with a phosphoric acid solution. The resulting HA crystals have a size of 10-20 nm in length, and a specific surface area of above 200 m^2/g.

The percentage of nano hydroxyapatite in the composition may range from about 10 to about 50% by weight, or from about 10 to about 20% by weight to the weight of the total scaffold composition.

The polymeric materials used in the present invention can be produced by conventional polymerization methods. Thus, the polymerization of the epsilon caprolactone monomers and the lactide monomers can be carried out in the melt phase or liquid phase as a conventional bulk polymerization by contacting the monomer at elevated temperature with a homogeneous catalyst. The amount of the catalyst is about 0.001 to 2 % calculated based on the volume of the epsilon caprolactone. By adjusting the monomer to catalyst ratio, it is possible to regulate the mechanical properties of the material and behaviour of the material in biological environment.

It is an advantageous aspect of the present invention that the scaffold demonstrates substantial new bone formation without the incorporation of bone morphogenetic protein - 2 (BMP-2) well known for its osteoinductive properties.

The scaffolds of the present invention may be cut and shaped to take a desired size and form. The scaffold is an osteoconductive and osteoinductive system. The osteoinductive part is the

composite and the osteoconductive part is the porous polymer scaffold which provides the mechanical support.

Another aspect of the present invention relates to a process for preparation of the nano-composite scaffold of the combination of Poly-L-Lactide-co-ε-caprolactone, (PLC)/gelatin/nano-hydroxyapatite (PGH) comprising the steps of:

a) Preparation of a polymer solution;
b) mixing the polymer solution with gelatin in a ratio of 3:1 in a solvent;
c) mixing a dispersion of hydroxyapatite powder with the PLC-Gel mixture obtained in step b);
d) electrospinning the PLC-Gel-HA polymer mixture; and
e) storing said scaffold under vacuum.

For the preparation of pure polymeric PLC scaffold, the polymer is dissolved in a suitable solvent. Suitable solvents which can be used for preparation of the polymer solution include, but are not limited to1,1,1,3,3,3-hexafluoro-2-propanol (HFP), tetrahydrofuran (TMF), dichloromethane, dimethylformamide (DMF), carbon tetrachloride, cyclohexanone.

Suitably, the concentration of polymer in the solution may range from about 10 to 15% w/v, more preferably the concentration is 12% w/v.

The polymer solution is then mixed with gelatin in a ratio of 3:1 by weight in a solvent. Suitable solvent for the purpose of the present invention being 1,1,1,3,3,3-hexafluoro-2-propanol (HFP).

The polymer mixture is suitably mixed at temperatures of 20 to 100 °C, or from 20 to 50 °C, more preferably at 25 to 35 °C for about 10 to 20 hours.

To prepare PLC-Gel-HA polymer mix, the PLC-Gel polymer mixture is blended with hydroxyapatite (HA). Suitably, the HA powder is dispersed in a solvent such as HFP and sonicated for about 30 min before mixing with the PLC-Gel solution.

The above PLC-Gel-HA polymer mix is subjected to electrospinning. In an embodiment, the polymer solution is loaded into a plastic syringe connected to a blunt end stainless steel hypodermic needle, preferably, 24 guage. The polymer filled syringe is loaded onto a syringe pump with the syringe needle tip connected to the positive output of a high voltage power supply while the aluminum foil covered collector is grounded. The parameters used for the electrospininng process may be: 12 kV voltage, 12.5 cm tip to collector distance, 1.5mL/h flow rate and 55-58% relative humidity.

The PLC/Gelatin/nano-hydroxyapatite scaffold in accordance to the present invention is prepared by the process of electrospinning followed by alternate soaking method, forms a nano sized poorly crystalline hydroxyapatite (HA) layer, uniformly distributed over the surface of polymer matrix. The presence of HA over surface of polymer matrix improves biocompatibility as well as provide osteophilic surface to scaffold for bonding with natural bone after implantation. Furthermore, presence of HA on surface of polymeric scaffold furnishes synthetic polymer scaffolds bioactive and osteoconductive and it changes chemistry as well topography at the surface.

This novel scaffold has been developed by electrospinning of Poly-L-Lactide-co-ε-caprolactone, (PLC) and a blend of Poly-L-Lactide-co-ε-caprolactone/gelatin (PLC/gel) followed by blending of hydroxyapatite (HA) mineralization via blending. The functional groups (COO^- and $-NH_2$) of gelatin in the PLC/gel scaffold facilitated the surface nucleation of HA as compared to the PLC scaffold.

The method of preparing the Poly-L-Lactide-co-ε-caprolactone, (PLC)/gelatin/hydroxyapatite composite scaffold in according to the present invention can be applied for the development

of various shapes and sizes of functional and biocompatible scaffolds for guided bone regeneration.

For making dense, non-porous polymer/mineral composites, the mixing is generally undertaken by melt extrusion or solvent/solution casting. Dense composites have high strengths but are lacking in osseointegration properties since the bone cells have less surface area to grow on. A porous structure is a better scaffold for bone cell growth. One method of making a porous structure is to add a so called porogen, i.e. a material, which supports the initial structure and is subsequently removed by washing or heating. A common porogen is sodium chloride, which is readily removed with water before the implantation.

According to one embodiment of the invention, the scaffold is prepared by electrospinning of Poly-L-Lactide-co-ε-caprolactone, (PLC) and a blend of Poly-L-Lactide-co-ε-caprolactone /gelatin (PLC/gel). Preferably, the ratio of PLC:gelatin is 3:1 by weight.

Microstructure of nanofibers produced according to the process of the present invention were observed by SEM and TEM. Crystal structure, chemical bonds and surface composition of both the scaffolds were characterized by XRD and FTIR respectively. HA acts as binder of gelatin in PLC/Gel/HA scaffold. All scaffolds were found to be blood biocompatible and cytocompatible during in-vitro experiments. PLC/Gel/HA scaffold displayed enhanced cellular adhesion and proliferation. Homogenous distribution of HA particles on electrospun fibers were observed by TEM and AFM. The proliferation and cellular adhesion of MG-63 cells were also enhanced on PLC/Gel/HA scaffold.

The material discussed above can be used in medical implants for promoting regeneration of biological tissue. Such a material can be further blended with other components, such as polylactide and polyglycolide. The scaffolds of the present invention affords better osteostimulation with lesser immune response attributed to the synergistic role of gelatin and HA. Particulatly, the scaffold becomes osteoconductive and osteophillic due to presence of n-

HA over the surface of scaffold, which facilitates human osteoblast like cells with increased osteoblastic activity in vitro.

Bone grafts prepared from the scaffolds of the present invention are used in: (i) maxillofacial surgery, (ii) in orthopaedics to repair defects created due to trauma, tumours and cysts, and (iii) in dentistry, where they are often used to cure periodontitis (bone loss at the tooth root). Many surgical procedures of the spine, pelvis and extremities require grafts. Bone grafts may also be needed in situations where healing may be difficult due to nicotine use, or the presence of diseases such as diabetes or autoimmune deficiencies. However, it has been found that the strength and processability, in particularly ductility, toughness and strength in combination with mouldability, of the material are such that it can also be used as the sole matrix component of the implant.

The following examples are provided to better illustrate the claimed invention and are not to be interpreted in any way as limiting the scope of the invention. All specific compositions, materials, and methods described below, in whole or in part, fall within the scope of the invention. These specific compositions, materials, and methods are not intended to limit the invention, but merely to illustrate specific embodiments falling within the scope of the invention. One skilled in the art may develop equivalent compositions, materials, and methods without the exercise of inventive capacity and without departing from the scope of the invention. It will be understood that many variations can be made in the procedures herein described while still remaining within the bounds of the invention. It is the intention of the inventors that such variations are included within the scope of the invention.

EXAMPLES

MATERIALS AND METHODS

We have procured the commercially available PLC as PURASORBR PLC-7015 from Purac Biomaterials of Gorinchem, Netherlands (PLC-7015 Material Patent status still unclear). Poly-L-lactic acid (PLLA) with average molecular weight 300,000 Da was purchased from Polysciences, Inc. Warrington, USA. Gelatin (Type A) from porcine skin and 1,1,1,3,3,3-

hexafluoro-2- propanol (HFP), trypsin-EDTA, penicillin-streptomycin, triton-X, Dimethyl sulfoxide (DMSO) and (3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (PBS) and Dulbecco's modified eagle's medium (DMEM) were purchased from Gibco-BRL (Grand Island,NY). Calcium chloride (CaCl2) and di-sodium hydrogen phosphate (Na2HPO4) were purchased from Merck, India. Water was distilled and deionized (DDW) using Milli-Q system (Millipore, MA, USA).

EXAMPLE 1:

Preparation of the Scaffold

Scaffolds were prepared by the process of electrospinning. For the preparation of pure polymeric PLC scaffold, a 12% w/v polymer solution was prepared and PLC-Gel (3:1 ratio, by weight) dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) by stirring overnight at room temperature. To prepare PLC-Gel-HA (HA is 40% of PCL-Gel, w/w) electrospun scaffolds, HA powder were weighed, dispersed in HFP, sonicated for 30 min, and then mixed with the 12 % w/v PLC-Gel solution. For electrospinning, the polymer solution was loaded into a 5 mL plastic syringe connected to a blunt end stainless steel hypodermic needle (24 Gauge) (BD, India). The polymer filled syringe was loaded onto a syringe pump (New Era Pump System Inc., USA) with the syringe needle tip connected to the positive output of a high voltage power supply (Gamma High Voltage, USA) while the aluminum foil covered collector was grounded. The parameters used for the electrospininng process were as follows: 12 kV voltage, 12.5 cm tip to collector distance, 1.5mL/h flow rate and 55-58% relative humidity.

Characterization of scaffold

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

The morphologies of the electrospun pure polymeric and composite scaffolds were examined by scanning electron microscope (Hitachi, S-3400 N, UK) at an accelerating voltage of 15kV. For SEM, the samples were cut into 5 x 5 mm squares, mounted on to sample stubs, and

sputter coated with palladium using a SC7640 Sputter Coater (Quorum Technologies Ltd, UK).

Microstructures of the fibers were studied by transmission electron microscopy (TEM, Tecnai 20, Philips FEI, Netherlands) at a voltage of 100kV. Fibers of PLC, PLC-Gel and PCL-Gel-HA were collected on TEM grids during the process of electrospinning.

Example-2

Hemocompatibility of scaffolds

Hemolysis assay

The hemocompatibility of all the scaffolds was evaluated by hemolysis assay described elsewhere. The method used to collect blood was as per institutional ethical guidelines. In brief 20 ml of whole blood was withdrawn from a healthy volunteer by ventipuncture into two 10 ml BD Vacutainer® Plus plastic plasma tubes which contain 150 USP units sodium heparin (spray-coated) anticoagulant. The erythrocytes were collected by centrifugation of heparinized blood at 1200 rpm for 15 min and then washed three times with normal saline. 1 ml of blood was added to all scaffolds which were first equilibrated in normal saline for 30 min at 37 °C. All samples were incubated for 1 hr at 37 °C in water bath. Thereafter, blood was centrifuged (1000 rcf, 10 min) and the absorbance of upper clear solution was measured by spectrophotometer at 540nm. 50% hematocrit was prepared by adding NSS to the erythrocytes. The hemolysis ratio (HR) was obtained by the equation

$$HR = \left(\frac{AS - AN}{AP - AN} \right) \times 100$$

Where AS, AP and AN are absorbance of sample supernatant, positive control and the negative control respectively.

Platelet adhesion assay

In brief, platelet-rich plasma (PRP) was obtained by centrifuging the fresh human blood (1500 rpm/min, 20 min). Scaffolds of 1 cm × 1 cm size were kept in 12-well tissue culture plate, equilibrated with PBS for 30 min at 37°C, and incubated with 1 mL of PRP for 2 h at 37°C. After incubation, scaffolds were washed with PBS to remove unattached platelets and

dehydrated with graded ethanol from 25% to 100%. Samples were dried overnight and sputter coated with gold and observed under SEM.

Example-3

Biological evaluation of the prepared scaffold

In vitro cell culture study

In vitro cell culture studies of the prepared scaffold have been carried out to assure that the scaffold is not toxic and biocompatible. MG63 cells were procured from National Centre for Cell sciences (NCCS), Pune, India and were cultured in Dulbecco's modified eagle's medium (DMEM) (Gibco, Invitrogen, USA) containing 2 mM L-glutamine (Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (Gibco, Invitrogen, USA) and 1% antibiotics-antimycotic solution (Sigma-Aldrich, USA), at 37°C in a humidified atmosphere of 5% CO_2. Prior to seeding, scaffolds were sterilized by dipping in 70% ethanol and kept under UV-light for 30 min, and washed three times with phosphate buffer saline (PBS) (Invitrogen, USA). MG63 cells grown in 25 cm^2 cell culture flask were detached by adding 700 µl of 0.25% trypsin and 0.2% EDTA (Invitrogen, USA). Detached cells were centrifuged, counted by trypan blue assay using a hemocytometer and seeded on the scaffold having a density of 1×10^4 cells/ml/well in 12 well tissue culture plates. Media was replenished after every day. FEG-SEM has been used to check the cell morphology attached to scaffold surface.

Cell adhesion: Scanning Electron Microscopy (SEM)

The cell attachment on to the scaffold was characterized by SEM. Sterilized scaffolds (1cm x 1cm) were kept in 24-well TCP and 1×10^4m MG63 were seeded on all scaffolds for a period of 2 and 5 days. At each time point, the cells on the samples were washed twice with PBS and fixed in 2.5% glutaraldehyde (Merck) for 2 h at room temperature, washed with PBS and dehydrated with graded ethanol from 25% to 100%. Samples were dried overnight, sputter coated with gold (Quorum Technologies Ltd, UK) and observed under SEM (Hitachi, S-3400 N, UK) at an accelerating voltage of 15 kV.

Cell infiltration: Confocal laser scanning microscopy (CLSM)

Cell infiltration inside the scaffolds was assessed by fluorescence microscopy after 5 days of culture. Scaffolds containing cells were washed twice with PBS. Cells were fixed in a 3.5% formaldehyde solution (Sigma-Aldrich) in PBS for 30 min at room temperature, washed with PBS twice Cell were then exposed to Fluorescein isothiocyanate-phalloidin (FITC-ph) (Invitrogen, CA, USA) (1:100 dil.) for 30 min in dark at room temperature to stain f-actin filament of cells to visualize the cell morphology and integrity. Samples were then dyed with Propidium iodide (PI, Sigma-Aldrich) for 1 h. After staining, samples were washed with PBS and mounted on to a glass-slide using a confocal laser scanning microscope (CLSM) (Olympus Fluoview, FV500, Tokyo, Japan).

RESULTS:

The results obtained after preparation and characterization of the scaffolds are analysed and discussed in this section. The results of characterization through the various techniques like SEM, TEM and AFM etc. are reported and discussed. Besides this, the results of biological assays such as cell attachment and cell infiltration are also described and analysed.

Morphology of electrospun scaffolds

The morphological study of electrospun scaffolds was carried out by SEM as shown in Figure 1. All scaffolds were found to resemble the extracellular matrix (ECM) of bone as they exhibited randomly oriented, uniform, non-woven morphology with interconnected pores. The composite fibers were prepared by blending hydroxyapatite (HA) particles in PLC-Gel solution. The ceramic particles were rarely traceable on PLC-Gel fibers when observed under SEM. HA particles were not widely present on surface of the fibers as they may be embedded into the polymer matrix and the self-assembled alignment stabilizes the hybrid polymer matrix. However, TEM images (Figure 2) confirms the presence of n-HA in composite matrix. The TEM dark field images show the crystalline mineral content brightly against the amorphous polymer background, thereby confirming the presence of n-HA crystallites.

The topography of scaffold surface plays a key role in cell attachment and proliferation, and therefore was studied at high resolution using AFM. AFM images confirmed the changes in

topography of scaffold after incorporation of gelatin in PLC scaffold (Figure 3a); small granular patterns are depicted on the surface of PLC/Gel scaffold (Figure 3b), whereas the PLC scaffold appeared flat (Figure 3a). AFM images (Figure 3c) also clearly indicated that changes occurred on the surface topography after mineralization of n-HA on PLC/Gel scaffold. Spiky ridges were observed on the PLC/Gel/HA scaffolds, whereas the PLC/Gel/HA surface showed larger granular structures. It could be attributed to efficient nucleation of n-HA in the presence of gelatin in the scaffold. Spiky ridges were observed on the PLC/Gel/HA scaffolds (Figure 3c), which was attributed to efficient surface nucleation of n-HA in the presence of gelatin in the scaffold.

Blood compatibility of scaffold

In vitro hemolysis assay was performed on PLC, PCL-Gel and PCL-Gel-HA scaffolds. Figure 4 shows the percentage hemolysis of all scaffolds. The PLC, PCL-Gel and PCL-Gel-HA scaffolds showed 0.67%, 0.41% and 0.30% hemolysis respectively. The percentage hemolysis of all scaffolds was under the permissible limit of 5%. This result confirms that addition of HA does not compromise the hemocompatibility of the scaffolds.

Adhesion, aggregation and activation of platelets are crucial steps in the process of thrombus formation. SEM micrographs were employed to assess platelet adhesion study on surface of all types of scaffolds, which are shown in Figure 5. The platelets adhered on the PLC and PLC/Gel scaffolds had round morphology. On PLC/Gel/HA scaffold, platelets were aggregated with flat morphology.

Cell adhesion: SEM

Adhesion of MG63 to scaffolds was analyzed by SEM on day 2 and day 5 of cell culture (Figure 6). SEM micrographs of MG63 cultured on PLC, PCL-Gel and PCL-Gel-HA exhibit the cells adhesion on all scaffolds. Initially at day 1, PCL-Gel-HA scaffold showed less number of cells attached as compared to other scaffolds (Figure 6). However, on day 5 all scaffolds were covered by a confluent layer of cells, indicating the efficiency of the scaffolds in supporting cell proliferation and growth (Figure 6).

Cellular infiltration: CLSM

The study of cell infiltration within electrospun nanofibrous scaffolds was carried out by confocal microscopy (Figure 7). Cell infiltration plays a key role in tissue engineered scaffolds and is governed by biochemical as well as biophysical cues provided by the surrounding scaffold. Figure 7 clearly indicates that the PLC/Gel/HA scaffold showed enhanced cell infiltration which could be due to the superior porosity of this scaffold as compared to others. Hence, cell infiltration as well as cell density was found to be improved in HA containing composite scaffolds.

We Claim:

1. A nano-composite scaffold for bone growth comprising: a Poly-L-Lactide-co-ε-caprolactone, (PLC), gelatin, and nanocrystalline hydroxyapatite (n-HA).

2. A nano composite scaffold as clamed in claim 1, wherein the Poly (L-lactide-co-ε-caprolactone, PLC) is made from L-lactide (PLA) and caprolactone (PCL) in a ratio of 70: 30.

3. The nano-composite scaffold as claimed in claim 1, wherein the ratio of PLC to gelatin is 3:1 by weight.

4. A nano composite scaffold as clamed in claim 1, wherein the weight of hydroxyapaptite in the composition is 40% to the total weight of PLC-Gel mixture.

5. A method of producing a scaffold for bone growth according to claim 1 comprising the steps of:
   a) preparation of a polymer solution;
   b) mixing the polymer solution with gelatin in a ratio of 3:1 in a solvent;
   c) mixing a dispersion of hydroxyapatite powder with the PLC-Gel mixture obtained in step b);
   d) electrospinning the PLC-Gel-HA polymer mixture; and
   e) storing said scaffold under vacuum.

6. The method as claimed in claim 5, wherein the electrospinning comprises:
   a) filling a syringe with the polymer solution and loading into a syringe pump;
   b) connecting the syringe needle tip to a positive output of a high voltage supply while the aluminium foil covered collector is grounded; and
   c) passing a voltage of about 12KV and maintaining a flow rate at 1.5mL/h.

7. The method as claimed in claim 5, wherein the polymer solution has a concentration of 12% w/v.

8. The method as claimed in claim 5, wherein the polymer solution is prepared in a solvent selected from 1,1,1,3,3,3-hexafluoro-2-propanol.

9. The method as claimed in claim 5, wherein the weight of hydroxyapaptite used is 40% to the total weight of PCL-Gel mixture.

10. The method as claimed in claim 8, wherein the solvent used for dispersing hydroxyapaptite is 1,1,1,3,3,3-hexafluoro-2-propanol.

Dated this 20 day of December 2013.

Indian Institute of Technology, Bombay
By their Agent & Attorney

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