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Nanofiber construct and method of preparing thereof

Field of the invention

The present invention relates to a nanofiber construct and method of preparing the same.

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Background of the invention

Different types of polymer fibers with nanometer scale diameter have been recently prepared by electrospinning method. As compared to the conventional polymer fibers with micrometer scale, nanofibers have a high surface area-to-
10 volume ratio. Hence, electrospun nanofibers appear to have better potential in several bioengineering applications, such as tissue regeneration, biosensors, recognition and filtration of viruses and drug molecules.

The interaction between nanofiber scaffolds and proliferation cells, like human osteoblasts, smooth muscle cells, mesenchymal stem cells and chondrocytes
15 have been investigated and the feasibility of nanometer scale polymer fibers as tissue scaffolds have been studied. However, nanometer scale dimension is not the only factor which encourages cell attachment and growth. It is known that human osteoblasts cannot attach to hydrophobic surfaces whereas endothelial cells can attach to hydrophobic surfaces.

20 Fujihara et al., Eight Japan International SAMPE Symposium and Exhibition, 2003, p.1213-6, disclosed the preparation of guided bone regeneration (GBR) membranes fabricated by polycaprolactone and calcium carbonate nanofibers with PLC to calcium carbonate 90:10 wt% ratio. They observed that calcium rich membranes were preferred in vivo conditions as they enhance
25 osteoconductivity of bone defects. However, they also found that the tensile property of these membranes decreased with the increase of amounts of

calcium carbonate particles. Accordingly, these membranes were not suitable for implant uses as they are mechanically not stable.

Accordingly, although prior art teaches electrospinning of polymer nanofibers, there exists a need to fabricate stable polymer composite nanofibers suitable as scaffolds for tissue engineering. In particular, there is a need to create improved composite nanofibrous membranes with appropriate composition and surface modification for application in tissue engineering in which cell attachment and growth are enhanced.

Summary of the invention

10 The present invention addresses the problems above, and in particular, provides a new composite nanofiber construct and method of producing the said construct.

According to a first aspect, the present invention provides a composite nanofiber construct comprising:

- 15 - at least a first nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle may be between the range of 99:1 and 10:90 weight percent; and
- 20 - at least a second nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle may be between the range of 100:0 and 70:30 weight percent.

The at least first nanofiber and the at least second nanofiber may have the same polymer to calcium salt nanoparticle ratio, or the at least first nanofiber and the at least second nanofiber may have a different polymer to calcium salt nanoparticle ratio. According to a particular aspect, the at least second nanofiber has a different polymer to calcium salt nanoparticle ratio from the at

least first nanofiber. In particular, the at least second nanofiber has a higher polymer to calcium salt nanoparticle ratio than the at least first nanofiber. For example, the polymer to calcium salt nanoparticle ratio of the at least first nanofiber may be 75:25 and the polymer to calcium salt nanoparticle ratio of the at least second nanofiber may be 95:5, 99:1 or 100:0. According to another particular aspect, the at least first nanofiber and the at least second nanofiber have the same polymer to calcium salt nanoparticle ratio. For example, the polymer to calcium salt nanoparticle ratio may be 75:25 weight percent.

The first nanofiber is also known as functionalised nanofiber. The second nanofiber is known as support nanofiber. The support nanofiber may comprise calcium salt. According to one aspect, the support nanofiber only comprises small amounts or traces of calcium salt. According to another aspect, the support nanofiber does not comprise calcium salt.

According to another aspect, the average diameter of the at least first nanofiber may be the same as or different from the average diameter of the at least second nanofiber. In particular, the average diameter of the at least first nanofiber is smaller than the average diameter of the at least second nanofiber.

The construct of the invention may be a single structure (for example, a layer) formed by the at least first nanofiber and the at least second nanofiber. Alternatively, the at least first nanofiber may form a first structure (for example, a first layer) and the at least second nanofiber may form a second structure (for example, a second layer), and wherein the first and second structures may be in contact with each other to form the construct. In particular, the first nanofiber and second nanofiber may be intertwined.

Accordingly, the structure may be a layer, or the structure may be such that the first and second structures are layers. According to one aspect, the construct according to the invention comprises at least a layer comprising functionalised (first) nanofiber(s) supported by at least a support layer comprising support

(second) nanofiber(s). According to another aspect, the functionalised nanofiber(s) and support nanofiber(s) are combined to form a single layer. For example, the functionalised nanofiber(s) and support nanofiber(s) are deposited simultaneously to form an intertwined layer. The construct according to the invention may comprise more than one of said layer.

The nanofiber(s), solvent mixture(s), construct(s), implant, kit and method according to the invention comprises the use of at least one polymer. Accordingly, a mixture of polymers may be used. The polymer may be a bioabsorbable polymer. The polymer may be selected from the group consisting of: polycaprolactone, polyethylene oxide, poly-L-lactic acid, polyglycolide, poly(DL-lactide), poly(L-lactide), polydioxanone, chitin, collagen either in its native form or cross-linked, poly(glutamic-co-leucine), poly-lactic-glycolide acid, poly(L-lactic acid-caprolactone) copolymer and blends, copolymers and terpolymers thereof. In particular, the polymer is ϵ -polycaprolactone.

The nanofiber(s), solvent mixture(s), construct(s), implant, kit and method according to the invention comprises the use of at least one calcium salt. Accordingly, more than one calcium salt or a mixture of calcium salts may be used in the present invention. The calcium salt may be selected from the group consisting of: calcium carbonate, calcium sulphate, calcium phosphate or hydroxyapatite. In particular, the calcium salt is calcium carbonate. However, more than one type of calcium salt or a mixture thereof may be used.

The construct may further comprise seeded cells. The cells may be selected from the group consisting of osteoblasts, endothelial cells, smooth muscle cells, mesenchymal stem cells, embryonic stem cells, chondroblasts, fibrocytes, fibroblasts and chondrocytes. In particular, the cells are human osteoblasts.

According to a further aspect, the construct is an implant or a scaffold.

The present invention also relates to the functionalization of nanofibers to make it suitable for use as tissue scaffolds and other applications in tissue engineering. Therefore, according to another further aspect, the construct may be surface functionalized. For example, the construct may be surface functionalized by polymer grafting and/or plasma treatment. The construct may also be surface functionalized by dipping and washing the construct in sodium hydroxide solution. In particular, the construct is surface functionalized by plasma treatment.

Accordingly, the present invention also provides the fabrication method and/or surface modification of composite nanofiber constructs. The invention enhances cell attachment and proliferation and by creating a suitable construct the mechanical strength of the construct can be appropriately tailored to meet the intended use.

The present invention, in particular, discloses a method of fabricating nanofibers suitable for use as scaffold for osteoblasts. This invention discloses methods of fabrication and/or surface modification of biodegradable and/or bioabsorbable polymer composite nanofibers. In particular, the construct according to the invention may have at least the following characteristics: (a) the composition of composite nanofibers can be tailored to the proliferation and attachment of different cell types; and (b) the mechanical properties and biochemical properties can be adjusted independently by electrospinning, surface functionalization and the addition of filler particles or nanoparticles such as calcium salt nanoparticles.

Accordingly, there is also provided a method of preparing the composite nanofiber construct according to any aspect of the invention, the method comprising the steps of:

- preparing at least a first nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 99:1 and 10:90 weight percent;

5 - preparing at least a second nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 100:0 and 70:30 weight percent; and

- preparing a composite nanofiber construct by contacting the first nanofiber with the second nanofiber.

10 The first nanofiber may be prepared by adding at least a calcium salt to at least a solvent and mixing the resulting mixture with the at least first polymer obtaining a first solvent mixture; and the second nanofiber may be prepared by adding at least a calcium salt to at least a solvent and mixing the resulted mixture with the at least second polymer obtaining a second solvent mixture. The second nanofiber may also be prepared by adding the second polymer to a solvent to obtain a second solvent mixture. In the latter case, no calcium salt is
15 added to the mixture.

Accordingly, the method of the invention may comprise the step of preparing a first solvent mixture and a second solvent mixture. The solvent of first and second solvent mixtures may be methanol and/or chloroform. Other suitable
20 solvents may also be used.

According to a particular aspect, the at least second nanofiber has a different polymer to calcium salt nanoparticle ratio from the at least first nanofiber. In particular, the at least second nanofiber has a higher polymer to calcium salt nanoparticle ratio than the at least first nanofiber. For example, the polymer to
25 calcium salt nanoparticle ratio of the at least first nanofiber may be 75:25 and the polymer to calcium salt nanoparticle ratio of the at least second nanofiber may be 100:0. According to another particular aspect, the at least first nanofiber

and the at least second nanofiber have the same polymer to calcium salt nanoparticle ratio. For example, the polymer to calcium salt nanoparticle ratio may be 75:25 weight percent. In particular, the ratio of polymer to calcium salt nanoparticle of the first nanofiber is 75:25 weight percent. In particular, the ratio
5 of polymer to calcium salt nanoparticle of the second nanofiber is 95:5 weight percent. Even more in particular, the ratio of polymer to calcium salt nanoparticle of the second nanofiber is 100:0 weight percent.

According to another aspect, the first solvent mixture may be provided to form the at least first nanofiber and the second solvent mixture may be provided to
10 form the at least second nanofiber.

According to another aspect, the average diameter of the at least first nanofiber may be the same as or different from the average diameter of the at least second nanofiber. In particular, the average diameter of the at least first nanofiber is smaller than the average diameter of the at least second nanofiber.

15 The first solvent mixture and second solvent mixture may be provided simultaneously such that the at least first nanofiber and the at least second nanofiber are intertwined. Alternatively, the first solvent mixture and second solvent mixture may be provided sequentially one after another such that the at least first nanofiber and the at least second nanofiber are separate from each
20 other. In particular, the first solvent mixture and second solvent mixture may be provided to form the at least first nanofiber and the at least second nanofiber respectively.

The method of the invention may further comprise the step of seeding the construct with cells. The cells may be selected from the group consisting of
25 osteoblasts, endothelial cells, smooth muscle cells, mesenchymal stem cells, embryonic stem cells, chondroblasts, fibrocytes, fibroblasts and chondrocytes. In particular, the cells are human osteoblasts.

The method of the invention may further comprise the step of surface functionalizing the construct. The step of surface functionalising may comprise polymer grafting and/or plasma treatment. The step of surface functionalizing may also comprise dipping and washing the construct in sodium hydroxide solution. In particular, the step of surface functionalising comprises plasma treatment of the construct. Other known methods of surface functionalization suitable for the purposes of the present invention are also encompassed by the construct and/or method of the present invention.

Therefore, a fabrication method and/or surface modification of polymer composite nanofiber construct(s) is provided. The method comprises the preparation of polymer solution comprising filler particles such as calcium salt particles, the principle of electrospinning method and preferably air-plasma treatment to enhance the hydrophilicity of composite nanofiber constructs. Additionally, nanofibers constructs are formed by electrospinning two or more types of nanofibers either simultaneously or sequentially in layers. In the composite nanofiber construct, the diameter and composition of one type of nanofiber may be adjusted for cell attachment and growth while the diameter and composition of the other may be adjusted for mechanical strength. In another type of composite nanofiber construct, the diameter and composition of each type of nanofiber is selected for cell attachment and proliferation for specific desired cell types. Other aspects, features and advantages of the invention will become apparent to those of ordinary skill in the art upon review of the description of specific embodiments of the invention. Calcium salt nanoparticles are added to the solvent mixture to result in composite nanofibers to enhance cell attachment, in particular osteoblast attachment. Such composite nanofibers are suitable for use as bone graft substitutes. The addition of calcium salt nanoparticles enhances the osteoconductive property of the nanofibers. Furthermore, human osteoblasts prefer a calcium rich environment. Composite nanofibers embedded with calcium phosphate can

enhance cell attachment and growth. Besides nanometer scale fiber constructs, functionalization (surface modification) of nanofibers is of paramount importance to promote better cell fiber interaction.

There is also provided a kit comprising the construct of the present invention. In particular, the kit comprises:

- at least a first nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 99:1 and 10:90 weight percent; and
- at least a second nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 100:0 and 70:30 weight percent.

The kit according to the invention may further comprise instructions for use(s) and/or application(s) of the construct, scaffold and/or implant according to the invention.

The present invention further provides a method of repairing fractured bone segment(s) in a subject, comprising the step of surgically implanting the construct of the present invention.

The present invention also provides a method of fabricating composite nanofibers constructs for use as tissue engineering scaffold and/or bone void fillers comprising the following steps:

- a) dispersing particles of calcium salt in a solvent;
- b) adding a polymer to the resultant mixture from (a) and agitating until the polymer is completely dissolved;
- c) the resultant polymer solution containing calcium salt particles of (b) is dispensed through one or more outlets from one or more dispensers at a

predetermined rate under controlled humidity and at a predetermined height separating the said discharge outlets from a collector plate;

d) a voltage is applied between the lowest point of the dispenser and a collector plate;

5 whereby application of voltage draws the resultant mixture (c) into fine elongations and with simultaneous evaporation of the solvent resulting in the deposition of composite nanofibers on the collector plate.

The method further includes drying of the said composite nanofiber constructs sufficient for all the solvent to evaporate. The solvent is a mixture of chloroform
10 and methanol. Further, the polymer is a bioabsorbable polymer. The polymer may be selected from lactone, polyethylene oxide, poly-L-lactic acid, poly-lactic-glycolide acid and poly (L-lactic acid – caprolactone) copolymer.

Further, the said calcium salt is any suitable combination of one or more calcium salt selected from the following: calcium carbonate, calcium sulphate,
15 calcium phosphate or hydroxyapatite. The nanoparticles of calcium salt is between 5 nm and 1000 nm in its largest dimension.

The said solvent may vary in composition from 95 weight percent chloroform and 5 weight percent methanol to 5 weight percent chloroform and 95 weight percent methanol. The mass of calcium salt nanoparticle may vary from 1g to
20 50 g per 100g of the said solvent. The mass of polycaprolactone may vary from 1 weight percent to 40 weight percent.

The dispenser in step (c) is any container with one or more outlets and wherein the outlet diameter ranges from 0.05 to 0.9 mm. In particular, the dispenser is a syringe connected to a hypodermic needle. The hypodermic needle may be a
25 #27 gauge. The method may further comprise a dispenser controller for controlling the dispensing of the resultant mixture of step (b). The dispensing rate may be between 0.1ml to 10ml per outlet per hour.

The predetermined height separating the discharge outlets from the collector plate may vary from 1mm to 1meter. There may be multiple dispensers connecting to one or more outlets. Further, each dispenser may dispense different formulations of the mixture from step (b). In particular, one or more dispenser dispenses bioabsorbable polymer without calcium salt.

The method may further include the step of air plasma treatment of the composite nanofibers. The method comprises the step of storing the composite nanofibers in aqueous solution until it is ready for use.

10 Brief description of the figures

Figure 1 (a) shows an electrospinning apparatus of polymer nanofibers with one dispenser and outlet.

Figure 1 (b) shows an electrospinning apparatus of polymer nanofibers with two dispensers and outlets.

15 Figure 2 shows a two-layer structure of composite nanofiber construct.

Figure 3 shows air-plasma treatment of composite nanofiber constructs.

Figure 4 shows SEM (Scanning Electron Microscope) photographs of (a) composite nanofiber construct comprising PCL and (b) composite composite nanofibers comprising PCL / CaCO₃.

20 Figure 5 shows EDX (Energy Disperse X-ray) mapping of (a) composite nanofiber construct comprising PCL and (b) composite nanofibers comprising PCL / CaCO₃ (PCL : CaCO₃ = 25 : 75).

Figure 6 shows osteoblast attachment manner on composite nanofiber scaffolds.

Figure 7 shows SEM photographs of PCL nanofibers made of (A) PCL 5 weight percent solution and (B) PCL 7.5 weight percent solution.

Figure 8 shows SEM photographs of PCL/CaCO₃ nanofibers (PCL:PCL/CaCO₃ = 25:75) made of (A) PCL 3 weight percent solution and (B) PCL 5 weight percent solution.

Figure 9 shows the absorbance intensity at 490 nm of TCPS and the construct against seeding time.

Figure 10 shows osteoblast attachment manner on the construct.

Figure 11 shows a two-layer structure of the construct to avoid rupture during cell seeding procedure. Two different composite nanofibers (i.e. GBR membrane (A) PCL:CaCO₃ = 75:25 and GBR membrane (B) PCL:CaCO₃ = 25:75) were prepared for osteoblast seeding.

Figure 12 shows the tensile stress-strain curve of nanofiber (B). Visible rupture was not recognized at 200% apparent strain.

15 Detailed description of the invention

The whole contents of any bibliographic reference is herein incorporated by reference.

According to a first aspect, the present invention provides a composite nanofiber construct comprising:

- at least a first nanofiber comprising at least a polymer and calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 99:1 and 10:90 weight percent; and
- at least a second nanofiber comprising at least a polymer and calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 100:0 and 70:30 weight percent.

It will be evident to a skilled person that the at least first nanofiber and the at least second nanofiber are distinguishable from each other. For example, the at least first nanofiber and the at least second nanofiber may have different physical and/or chemical characteristics. The physical and/or chemical characteristic may include, but is not limited to, polymer to calcium salt nanoparticle ratio, diameter, surface functionalization treatment and treatment to adapt the nanofiber to a particular function.

Nanofibers may comprise fibers ranging in diameter from approximately 1 nanometer (nm) (10^{-9} meters) to approximately 10000 nm. In particular, the fibers range in diameter from 10 nm to 10000 nm, preferably from 200 nm to 1500 nm.

According to a particular aspect, the average diameter of the section(s) of the at least first nanofiber may be the same as or different from the average diameter of the section(s) of the at least second nanofiber. In particular, the average diameter of the at least first nanofiber is smaller than the average diameter of the at least second nanofiber. The at least first nanofiber preferably has an average diameter between 10 and 1000 nm, more preferably between 20 and 500 nm, and even more preferably, 25 to 100 nm. In particular, the average diameter is about 50 nm, more in particular, 50 nm. The at least second nanofiber preferably has an average diameter between 10 and 1000 nm, more preferably between 20 and 500 nm, and even more preferably, 100 to 400 nm. In particular, the average diameter is about 300 nm, more in particular 300 nm.

The ratio of polymer to calcium salt nanoparticle of the at least first nanofiber may be between 99:1 to 10:90, 80:20 to 20:80, 75:25 to 25:75, 70:30 to 30:70, 60:40 and 40:60 or 50:50. In particular, the ratio of polymer to calcium salt nanoparticle of the first nanofiber is 70:30 weight percent. More in particular, the ratio of polymer to calcium salt nanoparticle of the first nanofiber is 75:25.

The ratio of polymer to calcium salt nanoparticle of the at least second nanofiber may be between 100:0 to 70:30, 95:5 to 80:20, 90:10 to 85:15 or 85:15 to 80:20. In particular, the ratio of polymer to calcium salt nanoparticle of the second nanofiber is 95:5 weight percent. More in particular, the ratio of polymer to calcium salt nanoparticle of the second nanofiber is 100:0 weight percent.

According to a particular aspect, the at least second nanofiber has a different polymer to calcium salt nanoparticle ratio from the at least first nanofiber. In particular, the at least second nanofiber has a higher polymer to calcium salt nanoparticle ratio than the at least first nanofiber. For example, the polymer to calcium salt nanoparticle ratio of the at least first nanofiber may be 75:25 weight percent and the polymer to calcium salt nanoparticle ratio of the at least second nanofiber may be 100:0 weight percent. According to another particular aspect, the at least first nanofiber and the at least second nanofiber have the same polymer to calcium salt nanoparticle ratio. For example, the polymer to calcium salt nanoparticle ratio may be 75:25 weight percent.

In particular, the at least first nanofiber and the at least second nanofiber have the same polymer to calcium salt nanoparticle ratio, but the average diameter of the at least first nanofiber and the at least second nanofiber are different. For example, the ratio of polymer to calcium salt nanoparticle in the at least first and second nanofibers is 75:25, and the average diameter of the at least first nanofiber is approximately 50 nm while the average diameter of the at least second nanofiber is approximately 300 nm. The at least second nanofiber provides mechanical support to the construct by virtue of its larger diameter.

The at least first nanofiber and the at least second nanofiber may form one structure or the at least first nanofiber may form a first structure and the at least second nanofiber may form a second structure, and wherein the first and

second structures may be in contact with each other. In particular, the first nanofiber and second nanofiber may be intertwined.

The structure may be a layer, or the structure may be such that the first and second structures are separate layers. According to one aspect, the construct
5 according to the invention comprises at least a layer comprising functionalised (first) nanofiber(s) supported by at least a support layer comprising support (second) nanofiber(s). According to another aspect, the functionalised nanobifer(s) and support nanofiber(s) are combined to form a single layer. For example, the functionalised nanofiber(s) and support nanofiber(s) are deposited
10 simultaneously to form an intertwined layer. The construct according to the invention may comprise more than one of said layer.

The polymer may be a bioabsorbable polymer. The polymer may be selected from the group consisting of: polycaprolactone, polyethylene oxide, poly-L-lactic acid, polygyycolide, poly(DL-lactide), poly(L-lactide), polydioxanone, chitin,
15 collagen either in its native form or cross-linked, poly(glutamic-co-leucine), poly-lactic-glycolide acid, poly(L-lactic acid-caprolactone) copolymer and blends, copolymers and terpolymers thereof. In particular, the polymer is ϵ -polycaprolactone. However, any suitable polymer may be used. Further, the at least first nanofiber and the at least second nanofiber may comprise one type of
20 polymer or a mixture of two or more polymers.

The present invention also provides that the calcium salt nanoparticle is selected from the group consisting of: calcium carbonate, calcium sulphate, calcium phosphate or hydroxyapatite. In particular, the calcium salt is calcium carbonate. However, any suitable calcium salt may be used. Further, the at
25 least first nanofiber and the at least second nanofiber may comprise one type of calcium salt or a mixture of two or more calcium salts.

The construct may further comprise seeded cells. The cells may be selected from the group consisting of osteoblasts, endothelial cells, smooth muscle cells,

mesenchymal stem cells, embryonic stem cells, chondroblasts, fibrocytes, fibroblasts and chondrocytes. In particular, the cells are human osteoblasts. However, any suitable cell may be used. Further, the construct may comprise more than one type of cell.

- 5 According to a further aspect, the construct is an implant or a scaffold. The construct may be used in various applications including, but not limited to, tissue engineering, bone graft substitute and/or periodontal regeneration. The construct may also be used for non-therapeutic and/or cosmetic purposes. It would be known to a skilled person how to use the construct. For example, the
- 10 construct may be used in, but not limited to, non-therapeutic and/or cosmetic periodontal purposes. It is important for an implant to be capable of both osteointegration and osteoconduction. Osteointegration refers to direct chemical bonding of a biomaterial to the surface of bone without an intervening layer of fibrous tissue. This bonding is referred to as the implant-bone interface. A
- 15 primary problem with skeletal implants is mobility. Osteoconduction refers to the ability of a biomaterial to sustain cell growth and proliferation over its surface while maintaining the cellular phenotype. For osteoblasts, the phenotype includes mineralization, collagen production, and protein synthesis. Normal osteoblast function is particularly important for porous implants that require
- 20 bone ingrowth for proper strength and adequate surface area for bone bonding. In addition, implants may be both biocompatible and biodegradable.

- According to a further aspect, the present invention also relates to the functionalization of nanofibers to make it suitable for use as tissue scaffolds and other applications in tissue engineering. Therefore, the present invention
- 25 provides a surface functionalised scaffold. For example, the construct may be surface functionalized by polymer grafting and/or plasma treatment. The construct may also be surface functionalized by dipping and washing the construct in sodium hydroxide solution. In particular, the construct is surface functionalized by plasma treatment.

Hydrophobicity of material is also necessary for cell attachment and proliferation. Plasma treatment is a useful method to modify the surface-chemical structure of polymers and its most apparent effect is modified wettability. According to Liston et al, *Plasma surface modification of polymers for improved adhesion: a critical review*, 1994, VSP BV Netherlands, plasma-produced polar chemical groups increase the surface energy of polymer and decrease in surface contact angle. Lee et al, *Cell adhesion and growth on polymer surfaces with hydroxyl groups prepared by water vapour plasma treatment*, Biomaterials, 1991, 12:443-8, investigated the interaction between plasma-modified polymers and ovary cell behaviour. It was shown that hydroxyl groups are mainly produced on the surface of polymers and this surface modification resulted in the decrease of surface contact angle which led to good adhesion and growing manners of cells.

Contact angle analysis characterises the wettability of a surface by measuring the surface tension of a solvent droplet at its interface with a homogenous surface. In more technical terms, contact angle measures the attraction or repulsion those droplet molecules experience towards the surface molecules. Any suitable method may be used for measuring the contact angle.

Further, the present invention also provides a method of preparing the composite nanofiber construct of the present invention, the method comprising the steps of:

- preparing at least a first nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 99:1 and 10:90 weight percent;

- preparing at least a second nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 100:0 and 70:30 weight percent; and

- preparing a composite nanofiber construct by contacting the first nanofiber with the second nanofiber.

The method according to the invention may further comprise the step of preparing a first solvent mixture and a second solvent mixture. The first solvent mixture may be used to prepare the at least first nanofiber and the second solvent mixture may be used to prepare the at least second nanofiber. The first and second solvent mixtures may comprise methanol and/or chloroform. However, the composition of the solvent mixtures may vary to suit the needs of the present invention. For example, any one of the solvents, or a combination thereof, as listed in Zheng-Ming Huang et al, *A review on polymer nanofibers by electrospinning and their applications in nanocomposites*, Composites Science and Technology, 2003, 63:2223-2253, may be used.

According to a further aspect, the ratio of polymer to calcium salt nanoparticle of the first nanofiber may be between 99:1 to 10:90, 80:20 to 20:80, 75:25 to 25:75, 70:30 to 30:70, 60:40 and 40:60 or 50:50. In particular, the ratio of polymer to calcium salt nanoparticle of the first nanofiber is 70:30 weight percent. Even more in particular, the ratio of polymer to calcium salt nanoparticle of the first nanofiber is 75:25.

According to a further aspect, the ratio of polymer to calcium salt nanoparticle of the second nanofiber may be between 100:0 to 70:30, 95:5 to 80:20, 90:10 to 85:15 or 85:15 to 80:20. In particular, the ratio of polymer to calcium salt nanoparticle of the second nanofiber is 95:5 weight percent. Even more in particular, the ratio of polymer to calcium salt nanoparticle of the second nanofiber is 100:0 weight percent.

According to a particular aspect, the at least second nanofiber has a different polymer to calcium salt nanoparticle ratio from the at least first nanofiber. In particular, the at least second nanofiber has a higher polymer to calcium salt nanoparticle ratio than the at least first nanofiber. For example, the polymer to

calcium salt nanoparticle ratio of the at least first nanofiber may be 75:25 weight percent and the polymer to calcium salt nanoparticle ratio of the at least second nanofiber may be 100:0 weight percent. According to another particular aspect, the at least first nanofiber and the at least second nanofiber have the same
 5 polymer to calcium salt nanoparticle ratio. For example, the polymer to calcium salt nanoparticle ratio may be 75:25 weight percent.

According to another aspect, the average diameter of the at least first nanofiber may be the same as or different from the average diameter of the at least second nanofiber. In particular, the average diameter of the at least first
 10 nanofiber is smaller than the average diameter of the at least second nanofiber.

The at least first nanofiber preferably has an average diameter between 10 and 1000 nm, more preferably between 20 and 500 nm, and even more preferably, 25 to 100 nm. In particular, the average diameter is about 50 nm, more in particular 50 nm. The at least second nanofiber preferably has an average
 15 diameter between 10 and 1000 nm, more preferably between 20 and 500 nm, and even more preferably, 100 to 400 nm. In particular, the average diameter is about 300 nm, more in particular 300 nm.

In particular, the at least first nanofiber and the at least second nanofiber have the same polymer to calcium salt nanoparticle ratio, but the average diameter of
 20 the at least first nanofiber and the at least second nanofiber are different. For example, the ratio of polymer to calcium salt nanoparticle in the at least first and second nanofibers is 75:25, and the average diameter of the at least first nanofiber is approximately 50 nm while the average diameter of the at least second nanofiber is approximately 300 nm.

25 The polymer may be a bioabsorbable polymer. The polymer may be selected from the group consisting of: polycaprolactone, polyethylene oxide, poly-L-lactic acid, polyglycolide, poly(DL-lactide), poly(L-lactide), polydioxanone, chitin, collagen either in its native form or cross-linked, poly(glutamic-co-leucine), poly-

lactic-glycolide acid, poly(L-lactic acid-caprolactone) copolymer and blends, copolymers and terpolymers thereof. In particular, the polymer is ϵ -polycaprolactone. However, any suitable polymer may be used. Further, the at least first nanofiber and the at least second nanofiber may comprise one type of polymer or a mixture of two or more polymers.

The present invention also provides that the at least calcium salt nanoparticle is selected from the group consisting of: calcium carbonate, calcium sulphate, calcium phosphate or hydroxyapatite. In particular, the calcium salt is calcium carbonate. However, any suitable calcium salt may be used. Further, the at least first nanofiber and the at least second nanofiber may comprise one type of calcium salt or a mixture of two or more calcium salts.

According to a particular aspect, the method of the present invention comprises electrospinning the at least first nanofiber and the at least second nanofiber. Electrospinning apparatus to make nanofibers generally consists of a high voltage power supply, a dispenser pump with feeding rate controller, a dispenser in the form of a syringe containing a solvent mixture, an outlet in the form of a needle with small diameter hole and a fiber collecting plate, as shown in Figure 1A. In the electrospinning process, a high voltage is used to create an electrically charged jet of polymer solution. Generally, a positive charge is applied to the solvent mixture at the outlet end while the fiber collector plate is grounded. Because of the high voltage, an intense electrical field is generated between the outlet and fiber collector plate. When the electrical force exceeds the surface tension of the solvent mixture, jets of solvent mixture are drawn towards to the fiber collecting plate. The solvent mixture jet is stretched to nanometer scale by electrical force and the solvent evaporates from the stream of solvent mixture jets to form solid nanofibers. Polymer nanofiber membranes are formed by the deposition of the nanofibers on the collector plate.

Figure 1A shows a dispenser filled with a solvent mixture. The feed rate of the dispenser is controlled by a dispenser pump. A flexible plastic tube connects the dispenser to an outlet, which can be in the form of a small orifice such as a small bore hypodermic needle. The outlet is usually clamped to a stand. Using a high-voltage power supply, voltage is applied to the outlet tip at room temperature with controlled humidity conditions. For example, the humidity is set to 30 to 40% to control the evaporation rate of the solvent. The electrically charged solvent mixture forms a Taylor cone from the tip of the outlet to the grounded collector plate at a fixed distance. The fine jet of electrically charged solvent mixture is drawn towards the grounded collector plate. The elongation of the solvent mixture jet results in decreasing diameter of the jet and travels towards the collector plate in a spiral fashion. During this travel, the solvent in the solvent mixture evaporates in the air and randomly oriented nanofibers are deposited on the collector plate. Generally, the mechanical properties of composite nanofibers are weaker than that of nanofibers which comprise added particles, such as calcium salt nanoparticles.

Depending on the composition of the calcium salt nanoparticles in the solvent mixtures, the formed nanofiber may be fragile and difficult to handle, particularly when a large amount of calcium salt nanoparticles are added. To enhance the handling characteristic of the nanofiber construct, a composite nanofiber structure may be fabricated by a number of methods as described below.

According to a further aspect, the first solvent mixture is provided to form the at least first nanofiber and the second solvent mixture is provided to form the at least second nanofiber. The first solvent mixture and second solvent mixture may be provided simultaneously such that the at least first nanofiber and the at least second nanofiber are intertwined. Alternatively, the first solvent mixture and second solvent mixture may be provided sequentially one after another such that the at least first nanofiber and the at least second nanofiber are separate from each other. In particular, the first solvent mixture and the second

solvent mixture are dispensed to form the at least first nanofiber and the at least second nanofiber respectively.

In the preparation of the composite nanofiber construct, in addition to the solvent mixture comprising a polymer and calcium salt nanoparticles, higher strength nanofibers with nanofibers containing less or no calcium salt nanoparticles are electrospun with the polymer nanofibers containing the desired ratio of polymer to calcium salt nanoparticles. According to a particular aspect, a composite nanofiber construct may be formed by depositing at least a nanofiber layer with the desired mechanical strength to form the mechanical supporting nanofiber layer, containing less or no calcium salt nanoparticles (see Figure 2). Alternatively, the desired mechanical strength may be achieved by preparing the second nanofiber having a larger average diameter than the first nanofiber. A functional nanofiber layer containing the desired ratio of polymer to calcium salt nanoparticles for enhanced cell adhesion and attachment is then electrospun onto the mechanical supporting nanofiber layer as shown in Figure 2. This bi-layer composite nanofiber construct consists of a functional composite nanofiber layer, in which the composition of the nanofiber has been designed for cell attachment and proliferation, and a mechanical supporting nanofiber layer, to provide mechanical support for the construct. In particular, the mechanical supporting nanofiber layer comprises the at least second nanofiber prepared from the second solvent mixture and the functional composite nanofiber layer comprises the at least first nanofiber prepared from the first solvent mixture. Even more in particular, the second solvent mixture contains less or no calcium salt nanoparticles.

The first and second solvent mixtures with calcium salt nanoparticles and with essentially no calcium salt nanoparticles respectively, are prepared prior to the electrospinning process. The calcium salt nanoparticles may be referred to as filler nanoparticles. These filler nanoparticles are such that they promote cell attachment, growth and proliferation. The choice of the filler nanoparticles

depends on the cell type desired. For example, the addition of calcium salt nanoparticles encourages the attachment and proliferation of osteoblast on the nanofibers.

The calcium salt nanoparticles are first dispersed in a particular solvent. This is followed by the addition of the polymer. The polymer pellet is dissolved in the solvent comprising the calcium salt nanoparticles. The solvent mixture is mixed using suitable methods to form the first solvent mixture. For example, the mixing may be carried out by a magnetic stirrer. Further, a second solvent mixture may be prepared in a similar manner, except little or no calcium salt nanoparticles are added to the solvent.

According to a particular aspect, the functional composite nanofibers comprising the at least one first nanofiber are electrospun simultaneously with the mechanical supporting nanofibers comprising the at least one second nanofiber, as seen in Figure 1B. This results in the formation of a composite nanofiber construct, in which the functional composite nanofibers are entwined with the mechanical supporting nanofibers. The electrospinning apparatus may have two or more dispensers connected to two or more outlets. By way of example, as shown in Figure 1B, dispenser A contains a solvent mixture suitable for electrospinning mechanical supporting nanofibers comprising the at least second nanofibers and dispenser B contains a solvent mixture suitable for electrospinning functional composite nanofibers comprising the at least first nanofibers. The solvent mixture in dispenser A may contain little or no calcium salt nanoparticles, while the solvent mixture in dispenser B may contain a desired ratio of polymer to calcium salt nanoparticles. The mechanical supporting nanofibers and the functional composite nanofibers are electrospun simultaneously to produce entwined nanofibers. The flow rate, outlet size and polymer concentration can be selected independently in each of the dispensers to produce nanofibers of the desired diameter. Further, the composition and diameter of the nanofibers may be selected to target the growth and

proliferation of different desired cell types. By way of example as shown in Figure 1B, dispenser A contains a solvent mixture suitable for electrospinning functional polymer nanofibers targeted at endothelial cell growth and dispenser B contains a solvent mixture suitable for electrospinning functional composite nanofibers targeted at smooth muscle cells growth. The different functional composite polymer nanofibers are electrospun simultaneously to produce entwined nanofibers such that the nanofibers are targeted at growing multiple cell types on a single construct. The flow rate, outlet size and polymer concentration may be independently selected in each of the dispensers to produce nanofibers of the desired diameter for the growth and proliferation of different cell types.

According to another particular aspect, a mechanical supporting nanofiber layer, comprising the at least second nanofiber, is first deposited on the collector plate by electrospinning, followed by the deposition of the functional composite nanofiber layer, comprising the at least first nanofiber, by electrospinning as shown in Figure 2.

The nanofiber constructs are dried overnight at room temperature under vacuumed conditions. The morphology of the at least first and second nanofibers may be influenced by various processing parameters such as: 1) viscosity of the solvent mixture determined by the ratio of polymer to calcium salt nanoparticles and other additives; 2) applied voltage to the electrospinning apparatus; 3) feed rate of the solvent mixture(s); 4) the distance between the outlet tip and the collector plate; 5) the inner diameter of the outlet; and 6) the humidity surrounding the electrospinning apparatus. Therefore, it is important to optimise the various parameters to prepare the desired nanofibers.

According to a further aspect, the method may further comprise the step of seeding the construct with cells. The cells may be selected from the group consisting of osteoblasts, endothelial cells, smooth muscle cells, mesenchymal

stem cells, embryonic stem cells, chondroblasts, fibrocytes, fibroblasts and chondrocytes. In particular, the cells are human osteoblasts. However, any suitable cell type may be used. Further, one type of cell type or a combination of two or more cell types may be used.

- 5 According to another further aspect, the method may further comprise the step of surface functionalizing the construct. The step of surface functionalising may comprise polymer grafting and/or plasma treatment. In particular, the step of surface functionalising comprises plasma treatment of the construct. Surface functionalization is necessary based on cell type and cell feature. Surface
- 10 functionalization of polymer nanofibers includes polymer grafting, plasma treatment and composite fabrication of nanofibers. Surface functionalization may also comprise the dipping and washing of the nanofibers or the construct in sodium hydroxide solution. Other known methods of surface functionalization suitable for the purposes of the present invention are also encompassed by the
- 15 construct and/or method of the present invention. The advantage of surface functionalization are as described above.

As shown in Figure 3, air-plasma treatment is applied to the fabricated nanofiber constructs to enhance their hydrophilicity. Plasma treatment is a useful method to modify the surface-chemical structure of the constructs to

20 enhance the wettability of the construct surface. Plasma treatment results in a high-energy condition producing hydroxyl and carboxyl groups on the surface of the construct. Plasma-produced polar groups increase the surface free energy of the construct, resulting in the decrease of the contact angle. Contact angle is as an estimate of bonding quality. It is desired to maintain the stability of the

25 plasma treated construct as the modified surface loses wettability with time. This is due to the combination of thermodynamical reorientation of polar groups or the reaction of residual free radicals. In order to avoid this undesirable loss of surface wettability, the constructs, after air-plasma treatment, are stored in aqueous solution. According to a particular aspect, the at least first and/or the at

least second nanofibers are electrospun on 13 mm by 13mm cover slips. Cover slips may then be placed on a glass slide, which are placed in a plasma cleaner chamber. Plasma discharge is applied to the samples for 10 minutes with the radio frequency power set to 30W under vacuum conditions.

- 5 According to another aspect, the present invention provides a kit comprising the construct of the present invention. The kit may further comprise a set of instructions on how the construct is to be used. In particular, the construct in the kit may be an implant or a scaffold. The kit according to the invention may further comprise instructions for use(s) and/or application(s) of the construct,
10 scaffold and/or implant according to the invention.

The present invention further provides a method of repairing fractured bone segments in a subject, comprising the step of surgically implanting the construct of the present invention. The subject may be a human or an animal. In particular, the subject is human.

- 15 The present invention also provides the use of the construct of the present invention in non-therapeutic and/or cosmetic applications. It would be known to a skilled person how to use the construct. For example, the construct may be used in non-therapeutic and/or cosmetic periodontal purposes. The present invention therefore provides a method of cosmetic surgery in, but not limited to,
20 a subject, comprising the step of implanting the construct according to any aspect of the present invention. In particular, the method of cosmetic surgery comprises periodontal surgery.

- Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by
25 way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russel, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2001).

Example 1

In the present invention, the composite nanofiber constructs were prepared by ϵ -polycaprolactone (PCL) nanofibers and a composite of PCL and calcium carbonate nanoparticles (CaCO_3) nanofibers with a particular weight ratio, i.e., PCL: CaCO_3 = 25:75 wt%). The materials used were PCL pellet (M_n = 80,000) purchased from Sigma-Aldrich Singapore Pte. Ltd., and CaCO_3 nanoparticles (average particle size = 40nm: cubic type) supplied from NanoMaterials Technology Pte. Ltd. Singapore. For PCL nanofibers, the PCL pellet was first dissolved in a mixture of solvent comprising 75wt% chloroform and 25wt% methanol. The concentration of PCL solution was 7.5wt% to ensure fine fiber morphology in the resulting nanofibers. For PCL / CaCO_3 composite nanofibers, CaCO_3 nanoparticles were first dissolved in a mixture of solvent and subsequently, the PCL pellet was dissolved. The concentration of PCL in the resulting mixture was 5wt%. The outlet was a needle with 0.21mm inner diameter. The feed rate of both solvent mixtures were fixed to 1.0ml/hour by a dispenser pump. Using a high-voltage power supply (Model: M826, Gamma High-Voltage Research, USA), 20kV voltage was applied to the outlet tip at room temperature and 30%-40% humidity conditions. The distance between the outlet tip and fiber collector plate was fixed to 130mm. The fabricated nonofiber constructs were dried overnight at room temperature under vacuumed conditionds. Figure 4 shows a photograph of PCL nanofibers and PCL / CaCO_3 composite nanofibers. The average fiber diameter of PCL nanofibers was 600 ± 230 nm while that of PCL / CaCO_3 composite nanofibers was 900 ± 450 nm.

Figure 5 shows the Energy Disperse X-ray (EDX) mapping of PCL / CaCO₃ composite nanofibers. While the element of calcium was not recognized on PCL nanofibers (Figure 5A), the presence of calcium was detected in composite nanofibers (Figure 5B). In present invention, the hydrophilicity of the composite nanofiber construct was modified by air plasma treatment. Table 1 shows that 10 minutes of air plasma treatment remarkably changed the wettability of both PCL nanofibers and PCL/CaCO₃ composite nanofibers.

	PCL nanofibers	PCL/CaCO ₃ nanofibers
Before	134°	139°
After	0°*	0°*

Table 1: Surface contact angle of PCL nanofibers and PCL/CaCO₃ composite nanofibers before and after 10 minutes of air-plasma treatment. * represents that a water drop gradually absorbed into the mesh.

After the composite nanofiber constructs were subjected to air plasma treatment for 10 minutes, osteoblast seeding procedure was subsequently conducted on the functional composite layer side. Human osteoblasts (hFOB1.19, catalog no: CRL-11372, ATCC, USA) were cultured on the composite nanofiber constructs. The cells were seeded onto 4 samples of scaffolds at a density of 25000 cells/cm². The seeding times of the cells were 1, 3 and 5 days. Figure 6 shows the cell attachment and proliferation manners observed on the composite nanofiber constructs. The PCL / CaCO₃ composite nanofibers were incorporated with osteoblasts. As seen in Figure 6A, granulates, which imply the sign of mineralization associated with differentiation, were observed on the surface of the attached cells. It is therefore likely that composite nanofibers show better bone formation and osteoconductivity under in vivo conditions.

Example 2

Please note that with reference to this example, GBR membrane (A) refers to nanofiber (A) and GBR membrane (B) refers to nanofiber (B).

2.1 Fabrication of composite nanofibrous construct

5 2.1.1 Electrospinning

In this example, composite nanofibrous constructs were designed by epsilon-polycaprolactone (PCL) nanofibers and PCL/CaCO₃ composite nanofibers with two different weight ratios (i.e. PCL:CaCO₃ = 75:25 wt% and 25:75 wt%). The materials used were PCL pellet (*M_n* = 80,000) purchased from Sigma-Aldrich
 10 Singapore Pte. Ltd., and CaCO₃ nanoparticles (average particle size of 40nm, cubic type) supplied by NanoMaterials Technology Pte. Ltd. Singapore.

For PCL nanofibers, the PCL pellet was first dissolved in a mixture of 75wt% chloroform and 25wt% methanol. In order to obtain fine fiber morphology, the concentration of PCL solution was varied in the range from 3wt% to 7.5wt%.

15 For PCL / CaCO₃ composite nanofibers, CaCO₃ nanoparticles were first dissolved in the mixed solvent and subsequently, the PCL pellet was dissolved. The concentration of PCL solution was also varied in the range from 3wt% to 7.5wt%.

As shown in Figure 1, the prepared PCL solution was placed in a syringe whose
 20 needle inner diameter size was 0.21mm. The feed rate of PCL solution was fixed to 1.0ml/hour by a syringe pump. Using a high-voltage power supply (Model: M826, Gamma High-Voltage Research, USA), 20kV voltage was applied to the needle tip at room temperature and 30%~40% humidity condition. Electrically charged polymer solution formed a Taylor cone from the tip of the
 25 needle to the ground collector plate with a fixed distance 130mm. During this process, the solvent evaporated in the air and randomly oriented nanofibrous

meshes, which were fabricated on the collector plate. The fabricated samples were dried for one night at room temperature under vacuumed conditions.

2.1.2 Morphology of electrospun nanofibers

Electrospun nanofibers were coated with gold using sputter coating and their morphology was observed under scanning electron microscope (SEM) (Model: JSM-5800LV, JEOL Pte., Ltd.). Energy Dispersion X-ray (EDX) analysis was also conducted under SEM to confirm the existence of CaCO_3 nanoparticles on the composite nanofibers. The average diameter of electrospun nanofibers was determined by measurement of 30 single nanofibers with the SEM image using image analysis software (Image J, National Institutes of Health, USA).

2.1.3 Plasma treatment

The nanofibers were air-plasma treated by electrode less radio frequency glow discharge plasma cleaner (Model: PDC-001, Harrick Scientific Corporation, USA). The samples were placed on a glass slide and were stably placed in the chamber of plasma cleaner. Plasma discharge was applied to the samples for 10 minutes with the radio frequency power set as 30W under vacuuming conditions.

2.1.4 Contact angle measurement

Surface contact angle was measured by contact angle machine (VCA Optima XE Video Contact Angle System, Crest Technology Pte Ltd., Singapore). A distilled water drop was put on five different sites of the nanofibers and the measured angles were averaged.

2.2 Mechanical characterization of nanofibers

The composite nanofibers were carefully cut into the rectangular dimension of 10mm width and 60mm length. Tensile test of the nanofibers was measured by

Instron 5848 Microtester with 10mm/min cross-head speed with a 40mm gauge length. Tensile stress of each membrane was calculated on the nominal cross sectional area of the tensile specimens (not on total area of nanofibers).

2.3 Osteoblast proliferation study

5 2.3.1 Osteoblast seeding

Human osteoblast (hFOB1.19, catalog no: CRL-11372, ATCC, USA) was cultured on composite nanofibers. Before cell seeding, sample preparation was conducted as follows. Nanofibers were fabricated on coverslips whose dimensions were 13mm by 13mm and the edges were stuck together using
 10 medical grade silicon adhesive for one night at room temperature under vacuumed conditions. The nanofibers on the coverslips were accordingly subjected to plasma treatment for 10 minutes and transferred to 24-well culture plates. The nanofibers were then sterilized using 70% ethanol solvent for 60 minutes under UV light and rinsed 3 times by phosphate buffer saline (PBS).
 15 The samples were then incubated in complete medium which contained Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 Ham (DMEM/F12 1:1 mixture: Gibco, USA), 10% fetal calf/bovine serum (FBS) and 1% penicillin-streptomycin for 12 hours at 34C° and 5% CO₂. Primary osteoblast culture was maintained in complete medium until 80% confluency and was passaged 3
 20 times. Osteoblast cells were then detached with 1% trypsin/EDTA and this was followed by centrifuge and re-suspension processes. The number of suspended cells were counted using a hemocytometer. The cells were seeded onto 4 samples of composite nanofibers at a density of 25000 [cells/cm²]. The seeding times of cells were 1, 3 and 5 days. At each time, 3 samples were used to
 25 measure the number of attached cells by MTS assay and 1 sample was chemically treated for SEM observation. For reference purposes, cells were also seeded to tissue culture polystyrene (TCPS) with the same seeding conditions.

2.3.2 MTS assay

The number of viable cells was measured by MTS assay (CellTiter 96^R Aqueous Assay). The principle mechanism of this assay is that metabolically active cells react with a tetrazolium salt in MTS agent to produce a soluble formazan dye which can be absorbed at 490nm wavelength. Each sample was rinsed 3 times with PBS, followed by incubation with MTS reagent in serum-free culture medium for 3 hours. Aliquots were then pipetted into 5 wells of a 96-well culture plate and the absorbance at 490nm of the content in each well was measured by spectrophotometric plate reader (FLUOstar OPTIMA, BMG lab technologies, Germany).

2.3.3 SEM observation

In order to observe the cell attachment manner on the composite nanofibers, chemical fixation of cells was carried out on each sample. After 1, 3 and 5 days of culture, a sample was rinsed twice with PBS and subsequently fixed in 2% glutaraldehyde for 1.5 hours. After that, a sample was rinsed with distilled water and then dehydrated with graded concentration of ethanol, i.e., 50%, 75%, 95% and 100% ethanol for 15 minutes each. Finally, a sample was treated with hexamethyldisilazane and kept in a fume hood for air drying. Dried samples were coated with gold using sputter coating.

2.4 Results

2.4.1 Morphology of electrospun nanofibers

The morphology of polymer nanofibers is influenced by various processing parameters such as: 1) viscosity of polymer solution determined by polymer concentration and additives; 2) applied voltage; 3) feeding rate of polymer solution; 4) distance between needle tip and collector; 5) needle inner diameter; and 6) humidity surrounding spinning apparatus. In this study, parameters

except 1) were fixed as follows; 2) voltage = 20kV, 3) feeding rate = 1.0ml/hour, 4) distance = 130mm, 5) inner diameter = 0.21mm, and 6) humidity = 30~40%.

Viscosity of polymer solution was varied by PCL concentration and the amount of CaCO_3 nano particles. In terms of PCL nanofibers, fiber formation was not observed with 3wt% PCL concentration. However, increased PCL concentration resulted in fiber formation with beads, as seen in Figure 7A. Fine fiber morphology without beads (see Figure 7B) was achieved with 7.5wt% PCL concentration. The average fiber diameter was 600 ± 230 nm. It must be noted that beaded nanofibers relatively indicate lower mechanical strength as compared to fine surface nanofibers. As the nanofibers require mechanical stability which can sustain surgery operation, formation of beaded fibers should be avoided. Similarly, fiber morphology of PCL/ CaCO_3 composite nanofibers (PCL: CaCO_3 = 25:75 wt%) was also investigated. Although PCL nanofiber formation was not achieved with 3wt% concentration, beaded fibers were fabricated on PCL/ CaCO_3 composite nanofibers with 3wt% concentration (Figure 8A). This was due to the viscosity increase by the addition of 3 times the amount of nanoparticles against that of PCL. When PCL concentration was increased to 5wt%, non-beaded fibers with granulated surface were formed (Figure 8B). In order to confirm attached granulation on fiber surface, Energy Disperse X-ray (EDX) mapping was conducted with SEM. While the element of calcium was not recognized on PCL nanofibers (Figure 5(a)), presence of calcium was detected in composite nanofibers (Figure 5(b)). Hence, it was confirmed that granulation existed on nanofibers when added CaCO_3 nano particles were added. The average fiber diameter of PCL/ CaCO_3 composite nanofibers was 900 ± 450 nm. Based on the above-mentioned results, PCL/ CaCO_3 composite nanofibers with weight ratio of (PCL: CaCO_3 = 75:25 wt%) was successfully fabricated with 7wt% PCL concentration. The average fiber diameter was 760 ± 190 nm.

2.4.2 Hydrophilicity of nanofibers

The hydrophilicity of the fabricated PCL and PCL/CaCO₃ composite nanofibers were investigated. Table 1 above shows that 10 minutes plasma treatment remarkably changed the wettability of both PCL and PCL/CaCO₃ composite nanofibers.

5 2.4.3 Design of composite nanofibers

With this respect, as seen in Figure 11, the PCL/CaCO₃ composite nanofibers were mechanically supported with PCL nanofibers which have a higher tensile strength. In this example, two different types of nanofibers were prepared, i.e., nanofiber (A): PCL:CaCO₃ = 75:25 wt% + PCL, nanofiber (B): PCL:CaCO₃ =
 10 25:75 wt% + PCL. An equal amount of solution was spun in each layer of the nanofiber construct. Figure 12 shows tensile behavior of nanofiber (B) and the nanofibers could be stretched at around 200% strain without visible rupture. Nanofibers (A) and (B) were subjected to plasma treatment for 10 minutes and subsequently osteoblast seeding procedure was conducted.

15 2.4.4 Osteoblast proliferation

Figure 9 shows absorbance intensity at 490nm of TCPS and, nanofiber (A) (PCL:CaCO₃ = 75:25 wt%) and nanofiber (B) (PCL:CaCO₃ = 25:75 wt%). Absorbance intensity of nanofiber (A) was similar level to that of TCPS and the values of nanofiber (A) and TCPS increased during 5 days seeding time.
 20 Although absorbance intensity of nanofiber (B) also increased for 5 days, its value was lower than the other two samples. As seen in Figure 10, nanofiber A had good osteoblast attachment at 1 day (Figure 10 (a)). The composite nanofibers incorporated the osteoblast (Figure 10(b)). For 5 days photo seen in Figure 10(c), granulates were found on the surface of a cell. Similar but much
 25 more granulations were observed in nanofiber (B) (Figures 10 (d) and (f)) at 1 day and 5 day. It was also observed that osteoblast incorporated into the composite nanofibers (Figure 10 (e)). Although a difference is seen on MTS assay data between nanofibers (A) and (B), no significant difference of cell

attachment was recognized under SEM observation. Therefore, when differentiation of osteoblast proceeds, proliferation rate simultaneously decreases. Hence, there may be a possibility that because of this osteoblast property, certain number of cells differentiated on nanofiber (B) which resulted in lower absorbance than nanofiber (A). Hence, for *in vivo* condition, nanofiber (B) may show better bone formation and osteoconductivity than nanofiber (A).

Claims:

1. A composite nanofiber construct comprising:
 - at least a first nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 99:1 and 10:90 weight percent; and
 - at least a second nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 100:0 and 70:30 weight percent.
2. The construct according to claim 1, wherein the at least first nanofiber and the at least second nanofiber have a different polymer to calcium salt nanoparticle ratio.
3. The construct according to claim 1, wherein the at least first nanofiber and the at least second nanofiber have the same polymer to calcium salt nanoparticle ratio.
4. The construct according to any one of claim 1 to 3, wherein the ratio of polymer to calcium salt nanoparticle of the first nanofiber is 75:25 weight percent.
5. The construct according to any one of claims 1 to 3, wherein the ratio of polymer to calcium salt nanoparticle of the second nanofiber is 95:5 weight percent.
6. The construct according to any one of claims 1 to 3, wherein the ratio of polymer to calcium salt nanoparticle of the second nanofiber is 100:0 weight percent.

7. The construct according to any one of claims 1 to 6, wherein the average diameter of the at least first nanofiber is smaller than the average diameter of the at least second nanofiber.
8. The construct according to any one of claims 1 to 7, wherein the at least first
5 nanofiber and the at least second nanofiber form one structure.
9. The construct according to any one of claims 1 to 7, wherein the at least first nanofiber forms a first structure and the at least second nanofiber forms a second structure, and wherein the first and second structures are in contact with each other.
10. The construct according to any one of claims 1 to 9, wherein the first
10 nanofiber and second nanofiber are intertwined.
11. The construct according to any one of claims 8 to 10, wherein the structure is a layer.
12. The construct according to any one of claims 9 to 11, wherein the first and
15 second structures are layers.
13. The composite nanofiber implant according to any one of claims 1 to 12, wherein the polymer is a bioabsorbable polymer.
14. The implant according to any one of claims 1 to 13, wherein the polymer is selected from the group consisting of: polycaprolactone, polyethylene oxide,
20 poly-L-lactic acid, polyglycolide, poly(DL-lactide), poly(L-lactide), polydioxanone, chitin, collagen in its native or cross-linked form, poly(glutamic-co-leucine), poly-lactic-glycolide acid, poly(L-lactic acid-caprolactone) copolymer and blends, copolymers and terpolymers thereof.
15. The construct according to any one of claims 1 to 14, wherein the polymer is
25 ϵ -polycaprolactone.

16. The construct according to any one of claims 1 to 15, wherein the at least one calcium salt nanoparticle is selected from the group consisting of: calcium carbonate, calcium sulphate, calcium phosphate, hydroxyapatite and a combination thereof.
- 5 17. The construct according to any one of claims 1 to 16, wherein the calcium salt is calcium carbonate.
18. The construct according to any one of claims 1 to 17, wherein the construct further comprises seeded cells.
- 10 19. The construct according to claim 18, wherein the cells are selected from the group consisting of osteoblasts, endothelial cells, smooth muscle cells, mesenchymal stem cells, embryonic stem cells, chondroblasts, fibrocytes, fibroblasts and chondrocytes.
20. The construct according to any one of claims 1 to 19, wherein the construct is an implant.
- 15 21. The construct according to any one of claims 1 to 20, wherein the construct is surface functionalized.
22. The construct according to claim 21, wherein the construct is surface functionalized by plasma treatment.
- 20 23. A method of preparing a composite nanofiber construct, the method comprising the steps of:
- preparing at least a first nanofiber comprising at least a polymer and at least a calcium salt nanoparticle, wherein the ratio of polymer to calcium salt nanoparticles is between the range of 99:1 and 10:90 weight percent;
 - preparing at least a second nanofiber comprising at least a polymer and at least a calcium salt nanoparticles, wherein the ratio of polymer to calcium
- 25

salt nanoparticle is between the range of 100:0 and 70:30 weight percent;
and

- preparing a composite nanofiber construct by contacting the first nanofiber with the second nanofiber.

5 24. The method according to claim 23, wherein the at least first nanofiber and the at least second nanofiber have the same or a different polymer to calcium salt nanoparticle ratio.

25. The method according to claim 23 or 24, wherein the ratio of polymer to calcium salt nanoparticle of the first nanofiber is 75:25 weight percent.

10 26. The method according to claim 23 or claim 24, wherein the ratio of polymer to calcium salt nanoparticle of the second nanofiber is 95:5 weight percent.

27. The method according to claim 23 or claim 24, wherein the ratio of polymer to calcium salt nanoparticle of the second nanofiber is 100:0 weight percent.

15 28. The method according to any one of claims 23 to 27, wherein the average diameter of the at least first nanofiber is smaller than the average diameter of the at least second nanofiber.

29. The method according to any one of claims 23 to 28, wherein the at least first nanofiber and the at least second nanofiber form one structure.

20 30. The method according to any one of claims 23 to 28, wherein the at least first nanofiber forms a first structure and the at least second nanofiber forms a second structure, and wherein the first and second structures are in contact with each other.

31. The method according to any one of claims 23 to 30, wherein the first nanofiber and second nanofiber are intertwined.

32. The method according to any one of claims 23 to 31, wherein the structure(s) is a layer.
33. The method according to claim 30 or claim 31, wherein the first and second structures are layers.
- 5 34. The method according to any one of claims 23 to 33, wherein the polymer is a bioabsorbable polymer.
35. The method according to any one of claims 23 to 34, wherein the polymer is selected from the group consisting of: polycaprolactone, polyethylene oxide, poly-L-lactic acid, polyglycolide, poly(DL-lactide), poly(L-lactide),
10 polydioxanone, chitin, collagen in its native or cross-linked form, poly(glutamic-co-leucine), poly-lactic-glycolide acid, poly(L-lactic acid-caprolactone) copolymer and blends, copolymers and terpolymers thereof.
36. The method according to any one of claims 23 to 35, wherein the polymer is ϵ -polycaprolactone.
- 15 37. The method according to any one of claims 23 to 36, wherein the calcium salt nanoparticle is selected from the group consisting of: calcium carbonate, calcium sulphate, calcium phosphate or hydroxyapatite.
38. The method according to any one of claims 23 to 37, wherein the calcium salt nanoparticle is calcium carbonate.
- 20 39. The method according to any one of claims 23 to 38, wherein the first nanofiber is prepared by adding calcium salt to a solvent and mixing the resulting mixture with the first polymer obtaining a first solvent mixture; and the second nanofiber is prepared by adding calcium salt to a solvent and mixing the resulted mixture with the second polymer obtaining a second
25 solvent mixture.

40. The method according to any one of claims 23 to 38, wherein the first nanofiber is prepared by adding calcium salt to a solvent and mixing the resulting mixture with the first polymer obtaining a first solvent mixture; and the second nanofiber is prepared by adding the second polymer to a solvent obtaining a second solvent mixture.
41. The method according to claim 39 or claim 40, wherein the first and second solvent mixtures comprise methanol and/or chloroform.
42. The method according to any one of claims 39 to 41, wherein the first solvent mixture is provided to form the at least first nanofiber and the second solvent mixture is provided to form the at least second nanofiber.
43. The method according to any one of claims 39 to 42, wherein the first solvent mixture and second solvent mixture are provided simultaneously, and wherein the at least first nanofiber and the at least second nanofiber are intertwined.
44. The method according to any one of claims 39 to 43, wherein the first solvent mixture and the second solvent mixture are provided consecutively, and wherein the at least first nanofiber and the at least second nanofiber are separate from each other.
45. The method according to any one of claims 23 to 44, wherein the method further comprises the step of surface functionalizing the construct.
46. The method according to claim 45, wherein the step of surface functionalizing the construct is by plasma treatment.
47. The method according to any one of claims 23 to 46, wherein the method further comprises the step of seeding the construct with cells.

48. The method according to claim 47, wherein cell is selected from the group consisting of: osteoblasts, endothelial cells, smooth muscle cells, mesenchymal stem cells, embryonic stem cells, chondroblasts, fibrocytes, fibroblasts and chondrocytes.
- 5 49. The method according to any one of claims 23 to 48, wherein the construct is an implant.
50. A kit comprising a composite nanofiber construct comprising:
- at least a first nanofiber comprising a polymer and calcium salt nanoparticles, wherein the ratio of polymer to calcium salt nanoparticle is
10 between the range of 99:1 and 10:90 weight percent; and
 - at least a second nanofiber comprising a polymer and calcium salt nanoparticles, wherein the ratio of polymer to calcium salt nanoparticle is between the range of 100:0 and 70:30 weight percent.
- 15 51. The kit according to claim 50, wherein the at least first nanofiber and the at least second nanofiber have the same or a different polymer to calcium salt nanoparticle ratio.
52. The kit according to claim 50 or claim 51, wherein the average diameter of the at least first nanofiber is smaller than the average diameter of the at least second nanofiber.
- 20 53. A method of cosmetic surgery in a subject, comprising the step of implanting the construct according to any one of claims 1 to 22.
54. The method according to claim 53, wherein the method of cosmetic surgery comprises periodontal surgery.