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(54) **Title**: PERIODONTAL TISSUE GRAFTS

(57) **Abstract**: The present invention relates to implantable decellularized biphasic periodontal tissue grafts, methods for their manufacture, and use of such grafts for the treatment of periodontal defects, periodontal diseases and periodontal tissue regeneration; said decellularized biphasic periodontal tissue graft comprises: a first polymeric scaffold comprising a plurality of first polymer fibers, and a second polymeric scaffold comprising a plurality of second polymer fibers; wherein said first and second polymeric scaffolds are interconnected; said first polymer fibers are arranged such that said first polymeric scaffold has a porous structure with an average first pore size, and said second polymer fibers are arranged such that said second polymeric scaffold has a porous structure with an average second pore size; said first polymer fibers are coated with an osteoblast-derived extracellular matrix, and said second polymer fibers are coated with a periodontal ligament cell-derived extracellular matrix.

PERIODONTAL TISSUE GRAFTS

Field

[0001] The present invention relates to implantable decellularized biphasic periodontal tissue grafts, methods for their manufacture, and use of such grafts for the treatment of periodontal defects, periodontal diseases and periodontal tissue regeneration.

Background

[0002] Periodontitis is a common chronic inflammatory disease that results in degradation of the supporting tissues around teeth, which if left untreated, can lead to tooth loss. It is characterized by significant extracellular matrix destruction of the alveolar bone, periodontal ligament and gingiva surrounding the tooth. Current treatments are aimed at controlling the inflammatory aspect of the disease but do not achieve regeneration of the lost tissues, thus resulting in inferior aesthetics and function. A major challenge is the periodontium's complex structure consisting of soft (gingiva, periodontal ligament) and hard (bone and cementum) tissues, and significant co-ordination is required between these different components in order for regeneration to occur. Indeed, periodontal regeneration requires the formation of periodontal ligament fibres and the insertion of these fibres into newly formed cementum on the root surface, as well as reconstitution of the adjacent resorbed alveolar bone. However, periodontal wound healing following conventional therapy results in repair by collagenous scar tissue and is accompanied by the apical migration of gingival epithelium between the gingival connective tissue and the root surface.

[0003] Dedicated surgical techniques are required in order to promote periodontal regeneration and the most widely utilized of these is based around the principles of Guided Tissue Regeneration (GTR). This technique utilizes barrier membranes to selectively promote the repopulation of the periodontal defect by cells capable of periodontal attachment regeneration (periodontal ligament cells, osteoblasts) on the root surface at the expense of those that do not (gingival epithelial cells). However, although this approach is conceptually sound and can be successful in ideal clinical scenarios, the clinical results have been unreliable and predictable regeneration remains elusive.

[0004] Cell sheet technology allowing the non-enzymatic harvesting of cultured cell with an intact extracellular matrix provides the opportunity to deliver periodontal ligament cells directly to the root surface. This approach has been successfully used to promote periodontal regeneration in a number of small and large animal models.

[0005] However, whilst tissue engineered cell sheet technology is promising, there are underlying limitations in reaching clinical practice, such as the reliance on the patient's own cells, as well as the need for cell culture facilities and associated technical expertise. The use of decellularized matrices has the potential to overcome these limitations and allow for the provision of an "off-the-shelf" tissue graft for use in periodontal tissue repair and/or regeneration.

Summary of Invention

[0006] The inventors have identified that improved repair and/or regeneration of periodontal tissue may be achieved using a biphasic tissue graft which has been pre populated with one or more cell populations and subsequently decellularized. In particular, the inventors have produced a biphasic tissue graft wherein each phase of the biphasic tissue graft is coated with an extracellular matrix particular to each phase, by populating the phases of a graft with a cell population followed by decellularization. This enables the simultaneous repopulation of each phase of the graft by a different tissue type thereby facilitating repair and/or regeneration of the periodontium's complex structure.

[0007] Embodiment 1: A decellularized biphasic periodontal tissue graft comprising: a first polymeric scaffold comprising a plurality of first polymer fibers, and a second polymeric scaffold comprising a plurality of second polymer fibers; wherein said first and second polymeric scaffolds are interconnected; said first polymer fibers are arranged such that said first polymeric scaffold has a porous structure with an average first pore size, and said second polymer fibers are arranged such that said second polymeric scaffold has a porous structure with an average second pore size; said first polymer fibers are coated with an osteoblast-derived extracellular matrix, and said second polymer fibers are coated with a periodontal ligament cell-derived extracellular matrix.

[0008] Embodiment 2: The tissue graft according to embodiment 1, wherein said average first pore size is greater than said average second pore size.

[0009] Embodiment 3: The tissue graft according to embodiment 1 or 2, wherein said first scaffold comprises a fixed deposition molded polymer or a melt-electrospun polymer, and said second scaffold comprises a melt-electrospun polymer or a solution-electrospun polymer.

[00010] Embodiment 4: The tissue graft according to any one of the preceding embodiments, wherein said first scaffold comprises a melt-electrospun polymer, and said second scaffold comprises a solution-electrospun polymer.

[00011] Embodiment 5: The tissue graft according to any one of the preceding embodiments, wherein said average first pore size is from about 100μm – 1000μm in diameter.

[00012] Embodiment 6: The tissue graft according to any one of the preceding embodiments, wherein said first polymer fibers comprise fibers from about 10μm – 500μm in diameter.

[00013] Embodiment 7: The tissue graft according to any one of the preceding embodiments, wherein said average second pore size is from about 1 – 10μm in diameter.

[00014] Embodiment 8: The tissue graft according to any one of the preceding embodiments, wherein said first polymer fibers and/or said second polymer fibers comprise fibers from about 500nm – 10μm in diameter.

[00015] Embodiment 9: The tissue graft according to any one of the preceding embodiments, wherein said first polymeric scaffold is coated with calcium phosphate.

[00016] Embodiment 10: The tissue graft according to any one of the preceding embodiments, wherein said first polymeric scaffold and/or second polymeric scaffold is coated with one or more anti-inflammatory agents and/or one or more antibacterial agents.

[00017] Embodiment 11: The tissue graft according to embodiment 10, wherein an external surface of said first polymeric scaffold is coated with a membrane comprising said one or more anti-inflammatory agents and/or one or more antibacterial agents.

[00018] Embodiment 12: The tissue graft according to embodiment 11, wherein the membrane is a non-porous occlusive scaffold.

[00019] Embodiment 13: The tissue graft according to any one of embodiments 10 to 12, wherein said one or more antibacterial agents is also an anti-inflammatory agent.

[00020] Embodiment 14: The tissue graft according to any one of embodiments 10 to 12, wherein said one or more antibacterial agents is selected from the group consisting of macrolides, β-lactam antibiotics, metronidazole and tetracyclines.

[00021] Embodiment 15: The tissue graft according to any one of embodiments 10 to 14, wherein said antibacterial agent is a macrolide.

[00022] Embodiment 16: The tissue graft according to embodiment 15, wherein said antibacterial agent is azithromycin.

[00023] Embodiment 17: The tissue graft according to embodiment 16, wherein said azithromycin is present in an amount selected from any one of about 0.7μg/mm^3 to about 3μg/mm^3, about 3μg/mm^3 to about 10μg/mm^3, about 10μg/mm^3 to about 20μg/mm^3, about 20μg to about 50μg/mm^3, about 50μg/mm^3 to about 100μg/mm^3, about 100μg/mm^3 to about 200μg, about 200μg/mm^3 to about 300μg/mm^3, about 300μg/mm^3 to about 400μg/mm^3, about 400μg/mm^3 to about 500μg/mm^3

[00024] Embodiment 18: The tissue graft according to embodiment 16, wherein said azithromycin is present in an amount of about 3μg/mm^3 to about 100μg/mm^3.

[00025] Embodiment 19: The tissue graft according to embodiment 16, wherein said azithromycin is present in an amount of about 20μg/mm^3.

[00026] Embodiment 20: The tissue graft according to embodiment 16, wherein said azithromycin is present in an amount of about 10μg/mm^3.

[00027] Embodiment 21: The tissue graft according to embodiment 16 wherein said azithromycin is present in an amount of about 5μg /mm^3.

[00028] Embodiment 22: The tissue graft according to any one of the preceding embodiments, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of biodegradable polymers.

[00029] Embodiment 23: The tissue graft according to any one of embodiments 1 to 21, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of non-biodegradable polymers.

[00030] Embodiment 24: The tissue graft according to any one of embodiments 1 to 21, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of polymers selected from the group consisting of: aliphatic polyesters, poly(amino acids), modified proteins, polydepsipeptides, copoly(ether-esters), polyurethanes, polyalkylenes oxalates, polyamides, poly(iminocarbonates), polyorthoesters, polyoxaesters, polyamidoesters, poly(ε-caprolactone)s, polyanhydrides, polyarylates, polyphosphazenes, polyhydroxyalkanoates, polysaccharides, modified polysaccharides, polycarbonates, polytyrosinecarbonates, polyorthocarbonates, poly(trimethylene carbonate), poly(phosphoester)s, polyglycolide, polylactides, polyhydroxybutyrates, polyhydroxyvalerates, polydioxanones, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(maleic anhydride), polyvinylalcohol, polyesteramides, polycyanoacrylates, polyfumarates, poly(ethylene glycol), polyoxaesters containing amine groups, poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(dioxanone)s, poly(alkylene alkylate)s, biopolymers, collagen, silk, chitosan, alginate, and a blend of two or more of the preceding polymers.

[00031] Embodiment 25: The tissue graft according to embodiment 24, wherein the first polymer fibers and/or the second polymer fibers are polycaprolactones.

[00032] Embodiment 26: The tissue graft according to any one of the preceding embodiments, wherein the osteoblast-derived and/or the periodontal ligament cell-derived extracellular matrix comprises any one or more of collagen, elastin, reticulin, fibronectin, laminin, adhesive glycoproteins, glycosaminoglycans (GAG), proteoglycans, chemoattractants, cytokines, and/or growth factors.

[00033] Embodiment 27: The tissue graft according to any one of the preceding embodiments, wherein the osteoblast-derived extracellular matrix comprises any one of more of collagen, proteoglycans, glycoproteins, non-collagenous proteins, osteocalcin, osteopontin, bone sialoprotein, and any molecule that may be found within the native bone extracellular matrix.

[00034] Embodiment 28: The tissue graft according to any one of the preceding embodiments, wherein the periodontal ligament cell-derived extracellular matrix comprises any one of more of

collagen, proteoglycans, glycoproteins, non-collagenous proteins, osteocalcin, osteopontin, bone sialoprotein, periostin, sclerostin, cementum attachment protein, and any molecule that may be found within the native periodontal ligament or cementum extracellular matrix.

[00035] Embodiment 29: A method of producing a decellularized biphasic periodontal tissue graft comprising: adjoining a first polymeric scaffold comprising a plurality of first polymer fibers and having a porous structure with an average first pore size to a second polymeric scaffold comprising a plurality of second polymer fibers and having a porous structure with an average second pore size; seeding said first scaffold with one or more osteoblasts and culturing the tissue graft in a tissue culture medium; seeding said second scaffold with one or more layers of a periodontal ligament cell layer and culturing the tissue graft in a tissue culture medium; and decellularizing the tissue graft after said seeding and culturing of the first and/or second polymeric scaffolds.

[00036] Embodiment 30: A method of producing a decellularized biphasic periodontal tissue graft comprising: laying a plurality of first polymer fibers on a surface to provide a first polymeric scaffold having a porous structure with an average first pore size; laying a plurality of second polymer fibers on a surface to provide a second polymeric scaffold having a porous structure with an average second pore size; adjoining said first and said second polymeric scaffolds; seeding said first scaffold with one or more osteoblasts and culturing the tissue graft in a tissue culture medium; seeding said second scaffold with one or more layers of a periodontal ligament cell layer and culturing the tissue graft in a tissue culture medium; and decellularizing the tissue graft after said seeding and culturing of the first and/or second polymeric scaffolds.

[00037] Embodiment 31: A method of producing a decellularized biphasic periodontal tissue graft comprising: laying a plurality of first polymer fibers on a surface to provide a first polymeric scaffold having a porous structure with an average first pore size; laying a plurality of second polymer fibers on said first polymeric scaffold to provide a second polymeric scaffold having a porous structure with an average second pore size, connected to said first polymeric scaffold; seeding said first scaffold with one or more osteoblasts and culturing the tissue graft in a tissue culture medium; seeding said second scaffold with one or more layers of a periodontal ligament cell layer and culturing the tissue graft in a tissue culture medium; and decellularizing the tissue graft after said seeding and culturing of the first and/or second polymeric scaffolds.

[00038] Embodiment 32: A method of producing a decellularized biphasic periodontal tissue graft comprising: laying a plurality of first polymer fibers on a surface to provide a first polymeric scaffold having a porous structure with an average first pore size; laying a plurality of second polymer fibers on a surface to provide a second polymeric scaffold having a porous structure with an average second pore size; adjoining said first and said second polymeric scaffolds; seeding said first scaffold with one or more osteoblast and culturing the tissue graft in a tissue culture medium; harvesting one or more layers of a periodontal ligament cell layer previously cultured in a tissue culture medium with said second scaffold; and decellularizing the tissue graft after said seeding and culturing of the first and/or second polymeric scaffolds.

[00039] Embodiment 33: The method according to any one of embodiments 29 to 32, wherein said laying of the first and/or said second polymer fibers is by electrospinning.

[00040] Embodiment 34: The method according to any one of embodiments 29 to 33, wherein said average first pore size is greater than said average second pore size.

[00041] Embodiment 35: The method according to any one of embodiments 29 to 33, wherein said average first pore size is from about 100μm – 1000 μm in diameter.

[00042] Embodiment 36: The method according to any one of embodiments 29 to 35, wherein said average second pore size is from about 1μm – 10μm in diameter.

[00043] Embodiment 37: The method according to any one of embodiments 29 to 35, wherein first and/or second polymer fibers comprise fibers from about 500 nm– 10μm in diameter.

[00044] Embodiment 38: The method according to any one of embodiments 29 to 37, further comprising the step of coating said first polymeric scaffold with calcium phosphate prior to seeding the first polymeric scaffold with osteoblasts.

[00045] Embodiment 39: The method according to any one of embodiments 29 to 38, further comprising the step of coating said first and/or second polymeric scaffold with one or more anti-inflammatory agents and/or one or more antibacterial agents.

[00046] Embodiment 40: The method according to embodiment 39, wherein said coating is performed prior to seeding the first polymeric scaffold with osteoblasts.

[00047] Embodiment 41: The method according to embodiment 39, wherein said coating comprises applying to an external surface of said first polymeric scaffold a membrane comprising said one or more anti-inflammatory agents and/or one or more antibacterial agents.

[00048] Embodiment 42: The method according to embodiment 41, wherein the membrane is a non-porous occlusive scaffold.

[00049] Embodiment 43: The method according to any one of embodiments 39 to 42, wherein said one or more antibacterial agents is also an anti-inflammatory agent.

[00050] Embodiment 44: The method according to any one of embodiments 39 to 42, wherein said one or more antibacterial agents is selected from the group consisting of macrolides, β-lactam antibiotics, metronidazole and tetracyclines.

[00051] Embodiment 45: The method according to any one of embodiments 39 to 44, wherein said antibacterial agent is a macrolide.

[00052] Embodiment 46: The method according to embodiment 45, wherein said antibacterial agent is azithromycin.

[00053] Embodiment 47: The method according to embodiment 46, wherein said azithromycin is present in an amount of about 0.7μg/mm^3 to about 3μg/mm^3, about 3μg/mm^3 to about 10μg/mm^3, about 10μg/mm^3 to about 20μg/mm^3, about 20μg to about 50μg/mm^3, about 50μg/mm^3 to about 100μg/mm^3, about 100μg/mm^3 to about 200μg, about 200μg/mm^3 to about 300μg/mm^3, about 300μg/mm^3 to about 400μg/mm^3, about 400μg/mm^3 to about 500μg/mm^3.

[00054] Embodiment 48: The method according to embodiment 46, wherein said azithromycin is present in an amount of about 3μg/mm^3 to about 100μg/mm^3.

[00055] Embodiment 49: The method according to embodiment 46, wherein azithromycin is present in an amount of about 20μg/mm^3.

[00056] Embodiment 50: The method according to embodiment 46, wherein said azithromycin is present in an amount of about 10μg/mm^3.

[00057] Embodiment 51: The method according to embodiment 6, wherein said azithromycin is present in an amount of about 5μg/mm^3

[00058] Embodiment 52: The method according to any one of embodiments 39, 40 or 42 to 51, wherein the scaffold is coated with said agent by: dissolving an amount of said agent in ethanol and applying to the tissue graft; incubating the graft for a suitable time period; evaporating the ethanol at room temperature; and washing the grafts after the evaporation of ethanol.

[00059] Embodiment 53: The method according to any one of embodiments 29 to 52, wherein said decellularizing comprises the steps of perfusing the tissue graft with a solution of NH_4OH solution and Triton X-100, perfusing the graft with a DNase I solution, and perfusing the graft with sterile water.

[00060] Embodiment 54: The method according to embodiment 53, wherein each perfusion step is performed for about 30 minutes to 2 hours.

[00061] Embodiment 55: The method according to embodiment 53 or 54, wherein the perfusing is bi-directional.

[00062] Embodiment 56: The method according to any one of embodiments 29 to 55, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of biodegradable polymers.

[00063] Embodiment 57: The method according to any one of embodiments 29 to 55, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of non-biodegradable polymers.

[00064] Embodiment 58: The method according to any one of embodiments 29 to 55, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of polymers selected from the group consisting of: aliphatic polyesters, poly(amino acids), modified proteins, polydepsipeptides, copoly(ether-esters), polyurethanes, polyalkylenes oxalates, polyamides, poly(iminocarbonates), polyorthoesters, polyoxaesters, polyamidoesters, poly(ε-caprolactone)s, polyanhydrides, polyarylates, polyphosphazenes, polyhydroxyalkanoates, polysaccharides, modified polysaccharides, polycarbonates, polytyrosinecarbonates, polyorthocarbonates, poly(trimethylene carbonate), poly(phosphoester)s, polyglycolide, polylactides,

polyhydroxybutyrates, polyhydroxyvalerates, polydioxanones, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(maleic anhydride), polyvinylalcohol, polyesteramides, polycyanoacrylates, polyfumarates, poly(ethylene glycol), polyoxaesters containing amine groups, poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(dioxanone)s, poly(alkylene alkylate)s, biopolymers, collagen, silk, chitosan, alginate, and a blend or copolymers of two or more of the preceding polymers.

[00065] Embodiment 59: The method according to embodiment 58, wherein the first polymer fibers and/or the second polymers are polycaprolactones.

[00066] Embodiment 60: The method according to any one of embodiments 29 to 59, further comprising a step of sterilizing the tissue graft.

[00067] Embodiment 61: The method according to embodiment 60, wherein said sterilization is via ethlylene oxide, UV irradiation or gamma irradiation.

[00068] Embodiment 62: A decellularized biphasic periodontal tissue graft obtained or obtainable by the method according to any one of embodiments 29 to 61.

[00069] Embodiment 63: A method of treating and/or repairing a periodontal defect in a subject in need thereof, comprising implanting a tissue graft according to any one of embodiments 1 to 28 into the periodontium of said subject.

[00070] Embodiment 64: A method of treating periodontal disease or degeneration in a subject in need thereof, comprising implanting a tissue graft according to any one of embodiments 1 to 28 into the periodontium of said subject.

[00071] Embodiment 65: A method of regenerating periodontal tissue in a subject comprising implanting a tissue graft according to any one of embodiments 1 to 28 into the periodontium of said subject.

[00072] Embodiment 66: A composition according to any one of embodiments 1 to 28 for use in treating and/or repairing a periodontal defect in a subject in need thereof.

[00073] Embodiment 67: A composition according to any one of embodiments 1 to 28 for use in treating periodontal disease in a subject in need thereof.

[00074] Embodiment 68: A composition according to any one of embodiments 1 to 28 for use in regenerating periodontal tissue in a subject.

[00075] Embodiment 69: Use of a composition according to any one of embodiments 1 to 28 in the manufacture of a medicament for treating and/or repairing a periodontal defect in a subject.

[00076] Embodiment 70: Use of a composition according to any one of embodiments 1 to 28 in the manufacture of a medicament for treating periodontal disease in a subject.

[00077] Embodiment 71: Use of a composition according to any one of embodiments 1 to 28 in the manufacture of a medicament for regenerating periodontal tissue in a subject.

[00078] Embodiment 72: The method according to any one of embodiments 63 to 65, the composition according to any one of embodiments 66 to 68, or the use according to any one of embodiments 69 to 71, wherein the subject is a human subject.

[00079] Embodiment 73: The method according to any one of embodiments 63 to 65, wherein implanting a tissue graft comprises excising a portion of the gingival tissue of the subject to access bone within said periodontium and placing said tissue graft on a surface of said bone.

Brief Description of Drawings

[00080] **Figure 1** shows the fabrication of the biphasic tissue graft. A) fabrication scheme, B) and C) cross-sectional views of the biphasic tissue graft by scanning electron microscopy showing the fusion of the electrospun fibers onto the FDM component. (D –H) shows the fabrication of abiphasic tissue graft using solution-electrospun scaffold interconnected with a melt-electrospun scaffold.

[00081] **Figure 2** shows in vitro 2D culture of the periodontal ligament cells (a-d) and osteoblasts (e-h). a) and e) optical microscopy, b) and f) alizarin red S staining and semi-quantification, c) and g) cell proliferation as measured by DNA content assay, d) and h) alkaline

phosphatase activity measured from the media Bars and stars show significant differences as measured by one way ANOVA ($p < 0.05$)

[00082] **Figure 3** shows DNA content of osteoblasts within the biphasic tissue graft. Bars and stars show significant differences as measured by one way ANOVA ($p < 0.05$).

[00083] **Figure 4** shows SEM of the osteoblast-seeded biphasic tissue graft. The osteo-induced otsteoblasts gradually filled the pores of the bone compartment.

[00084] **Figure 5** shows confocal imaging of the osteoblast seeded biphasic tissue grafts. The nuclei are stained blue and the actin filaments red.

[00085] **Figure 6** shows multiple cell sheets onto the electrospun membrane (periodontal component) of the biphasic tissue graft. a) and b) anchorage points on the periodontal compartment, c) live dead assay, green staining indicates living cells as opposed to red which indicates dead cells , d) morphology of the last cell sheet, e) thickness of the three cell sheets.

[00086] **Figure 7** shows Description of biphasic tissue graft assembling onto the dentin block and illustration of the subcutaneous implantation in athymic rats.

[00087] **Figure 8** shows micro-CT of the biphasic tissue grafts 8 weeks post-implantation in a subcutaneous athymic rat model. Bars show the statistical difference between control (empty group) and osteoblast seeded scaffold (OB and OB induced)

[00088] **Figure 9** shows histological findings a)-d) Alkaline Phosphatase staining of the bone compartment. a) negative control staining, b) empty scaffold, c) osteoblasts seeded scaffold, d) osteoblasts induced scaffold, e)-r) periondontal compartment, e)-h) and i)-l) representative H&E and Azan staining at the interface with the dentin block in the presence or absence of multiple cell sheets, m)-r) CEMP1 staining at the interface with the dentin in the presence and absence of cell sheets. Black arrows show the location of the positive CEMP1 staining.

[00089] **Figure 10** shows a graphical illustration of the biomimetic coating procedure, the fabrication of the biphasic scaffold, cell seeding, subsequent in vitro culture and in vivo implantation of the cellular constructs. a) and b) harvesting and placement of cell sheets on the periodontal compartment. c) and d) positioning of the construct to a dentin block.

[00090] **Figure 11** shows Scanning Electron Microscopy and X-ray diffraction analysis of scaffolds. a) and c) Cross-sectional views of scaffolds. b) CaP-coated surface of FDM scaffold. d) Melt electrospun mesh. e) X-ray diffraction analysis of surface of non-coated and CaP-coated scaffold strut. * is hydroxyapatite and # is DCPD.

[00091] **Figure 12** shows Confocal laser microscopy a) and scanning electron microscopy images b) of the seeded scaffolds after 6 weeks of in vitro culture under four different conditions.. N-N - non coated scaffold cultured in basal medium, N-O - non coated scaffold cultured in osteogenic medium, CaP-N – calcium phosphate-coated scaffold cultured in basal medium, CaP-O – calcium phosphate-coated scaffold cultured in osteogenic medium.

[00092] **Figure 13** shows a biological characterization. A) DNA content versus time in culture. B) DNA content versus experimental groups. C) Normalized ALP activity versus time in culture. D) Normalized ALP activity versus experimental groups. E) In vitro volume of mineralization as measured by micro-CT analysis F) Mineralization density. a, b and * indicate statistical significance ($p<0.05$). In graph E) all values are statistically different ($p<0.05$). N-N: non coated scaffold cultured in basal medium, N-O: non coated scaffold cultured in osteogenic medium, CaP-N: calcium phosphate-coated scaffold cultured in basal medium, CaP-O: calcium phosphate-coated scaffold cultured in osteogenic medium.

[00093] **Figure 14** shows a Micro-CT analysis 8 weeks post-implantation. A) Bone volume and B) bone density. Same letters indicate statistical significance between the groups ($p<0.05$). C) Representative reconstruction of constructs from each group. N-N: non coated scaffold cultured in basal medium, N-O: non coated scaffold cultured in osteogenic medium, CaP-N: calcium phosphate-coated scaffold cultured in basal medium, CaP-O: calcium phosphate-coated scaffold cultured in osteogenic medium.

[00094] **Figure 15** shows Representative H&E (a-c) and immune (d) staining images of implanted biphasic scaffolds. a) Reduced tissue attachment on the dentin without cell sheets. b) Bone formation in the CaP-coated samples cultured in osteogenic media prior to implantation. d) Representative section depicting the vascularization in the periodontal compartment. c) Tissue orientation provided by the periodontal compartment architecture. DB- dentin block, BO- bone, VE- vessel, SC- Spaces formed by FDM scaffold's struts, MES indicates melt electrospun

scaffold and thin arrows indicate single melt electrospun fibers. Triangular arrows indicate periodontal ligament. Scales are 100 μm.

[00095] **Figure 16** shows harvesting of fresh Human periodontal ligament (HPDL) cell sheet and scanning electron microscopy (SEM) showing fresh and decellularized PDL sheet. (A) PCL scaffold of 5 mm diameter, after NH4OH treatment. (B) Fresh HPDL cell sheet attached to sterile PCL scaffold. (C) SEM image of decellularized PDL sheet on top of PCL scaffold. (D) Different SEM magnification of fresh (I-III) and decellularized (IV-VI) sheet.

[00096] **Figure 17** shows immunostaining of human collagen type I and fibronectin. (A-D) Staining of cell monolayers on coverslips; (E-H) Staining of mature cell sheet – polycaprolactone constructs. Nuclei (DAPI) in blue, Actin filaments (Phalloidin) in red, human collagen type I and human Fibronectin in grey.

[00097] **Figure 18** shows comparison of DNA amounts, growth factor concentrations and collagen content of fresh and decellularized periodontal ligament cell sheet constructs. (A) DNA content before and after decellularization. (B) Growth factors retained in fresh and decellularized sheet, Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF). (C) Collagen quantification showing preservation of collagen content after decellularization process.

[00098] **Figure 19** shows recellularization potential of the decellularized sheet after seeding with allogenic hPDL cells after 3, 7 and 21 days respectively. (A) Confocal imaging and immunostaining of human collagen type I (grey), Nuclei (blue), actin filaments (red). (B) SEM showing hPDL cells at different time points. (C) DNA quantification showing cell proliferation over 21 days.

[00099] **Figure 20** shows SEM images of PCL alone (A), PCL loaded with 1mg of azithromycin (B), Calcium phosphate coated PCL (PCL-CaP) (C) and PCL-CaP loaded with 1mg of azithromycin (D). When loaded on to PCL membranes azithromycin aggregates and forms a flower like shape on the other hand when coated onto calcium phosphate coated PCL membranes gets uniformly distributed across the membrane. (E) Encapsulation efficiency of azithromycin loaded on to PCL and calcium phosphate coated PCL (PCL-CaP). $n=4$, $p<0.05$. (F) Cumulative release of azithromycin from PCL and calcium phosphate coated PCL (PCL-CaP) achieved as a continuous release over a period of 14days ($n=3$).

[000100] **Figure 21** shows growth inhibition of staphylococcus aureus after 20 hour incubation with 1mg azithromycin containing (A) PCL (B) PCL-CaP electrospun membranes after 0, 7 and 14 days release from PBS at 37°C.

[000101] **Figure 22** shows the loading efficiency and release profile of high dose (A),(B) and low dose (C),(D) azithromycin loaded on PCL or PCL-CaP electrospun membranes .

[000102] **Figure 23** shows the contact angle measurement of PCL and PCL-CaP membranes loaded with different doses of azithromycin (A) and (B);Zeta Potential and pH of PCL and PCL-CaP membranes with or without azithromycin (5mg) (C); XRD spectrum of PCL and PCL-CaP membranes with or without azithromycin (D); and FTIR spectrum of PCL and PCL-CaP membranes with or without azithromycin.

[000103] **Figure 24** shows growth inhibition of *Staphylococcus aureus* after 20 hour incubation with 1mg (a), 500µg (b) and 50µg (c) azithromycin containing PCL-CaP electrospun membranes after 0,1,3,5 and 7 days release from PBS at 37°C.

[000104] **Figure 25** showslive dead assay on human osteoblast cultured with various doses of azithromycin coated PCL-CaP membranes (a) control group with PCL-CaP membranes alone, PCL-CaP membranes with 10µg (b), 50µg (c), 100µg (d), 250µg (e), 500µg) of azithromycin

[000105] **Figure 26** shows haematoxylin and eosin staining of PCL-CaP membranes loaded with 0µg (a), 100µg (b), 250µg (c), 500µg (d), 750µg (e) and 1mg (f), percentage of soft tissue attachment and integration (g) one week post-implantation in Sprague Dawley rats (n=3),* ($p<0.05$) when compared to 100µg and 250µg.

[000106] **Figure 27** shows haematoxylin and eosin staining of PCL-CaP membranes loaded with 0µg (a), 100µg (b), 250µg (c), 500µg (d), 750µg (e) and 1mg (f), percentage of soft tissue integration (g) four week post-implantation in Sprague Dawley rats (n=3).

[000107] **Figure 28** shows an experimental design for 3D tissue imaging and design. A: CT-scan of the bone defect. B and C: utilization of CAD/CAM technologies for fabricating a patient specific bilayered scaffold through Fused Deposition Modelling and Melt electrospinning writing. D: morphology of the bilayered scaffold by Scanning Electron Microscopy. E: Placement of the construct over the jaw bone defect to enhance *in vivo* bone formation.

Description of Embodiments

[000108] **Definitions**

[000109] As used herein, a "graft" will be understood to mean the device to be implanted in a subject during a medical grafting procedure.

[000110] As used herein, "decellularized" refers to an entity (e.g.,a bi-phasic tissue graft or scaffold etc.) that underwent a decellularization process (i.e., the removal of cells from the graft) and is thus devoid or substantially devoid of any cellular components, excluding extracellular matrix or other associated biomolecules.

[000111] The phrase "substantially devoid of any cellular components" as used herein refers to a status of more than 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, (e.g., 100 %) devoid of cellular components present incorresponding natural (e.g., native) tissue. As used herein, the phrase "cellular components" refers to cell membrane components or intracellular components which make up the cell. Examples of cellular components include cell structures (e.g., organelles) or molecules comprised in same. Examples of such include, but are not limited to, cell nuclei, nucleic acids, residual nucleic acids (e.g., fragmented nucleic acid sequences), cell membranes and/or residual cell membranes (e.g., fragmented membranes) which are present in cells of the tissue. It will be appreciated that due to the removal of all cellular components from an entity, such an entity may not induce an immunological response when implanted in a subject.

[000112] As used herein, "polymer" means a chemical compound or mixture of compounds formed by polymerization and including repeating structural units. Polymers may be constructed in multiple forms and compositions, or combinations of compositions.

[000113] The polymers described herein may be prepared by synthetic or natural methods. However, the method preferably provides the desired polymer in a form sufficiently pure for use as an implantable material. The polymer is preferred not to contain any undesirable residues or impurities which could elicit an undesirable response either in vitro in the case of a cell-seeded construct, or in vivo.

[000114] The polymers may be prepared from any combination of monomeric units. These units may be capable of biodegrading in vivo to nontoxic compounds, which can optionally be excreted or further metabolized. The combination of units in the polymer should be biocompatible to minimise or avoid any undesirable biological response upon implantation. The polymer may be biodegraded in vivo by any means, including hydrolysis, enzymatic attack, a cell-mediated process, or by any other biologically mediated process. It is considered desirable for tissue engineering applications that a polymer scaffold as described herein can serve as a transitional construct, and thus be fully degraded once the new tissue is able to take over the function of the scaffold. Since the rates at which different new tissues are likely to be able to assume their new function will vary, it is desirable to have polymers with a range of degradation rates as well as a range of different properties. Generally, however, preferred polymers may degrade in a matter of weeks to months, preferably less than one year, rather than several years.

[000115] The mechanical properties of the polymer may be designed to meet the needs of the particular tissue engineering application. Thus, according to the methods described herein for preparing bioabsorbable biocompatible polymers, the monomeric units can be selected to provide upon combination of the correct ratios of these monomeric units the desired property or property set. If necessary, the monomeric units may be combined in a specific order as in, for example, a block copolymer, or alternatively they can be assembled in a random manner. They may also be prepared with different molecular weights to achieve the desired performance.

[000116] As used herein, the terms “extracellular matrix” and “ECM” refer to a scaffolding for cell growth. The ECMs may include mixtures of structural and non-structural biomolecules, including, but not limited to, collagens, elastins, laminins, glycosaminoglycans, proteoglycans, antimicrobials, chemoattractants, cytokines, and growth factors.

[000117] As used herein, the term “-derived extracellular matrix” refers to the extracellular matrix which is produced, secreted, supplied or deposited by the cell from which the ECM is said to derive. For example, an osteoblast-derived extracellular matrix refers to an ECM which has been produced, secreted, supplied or deposited by one or more osteoblasts. A periodontal ligament cell-derived extracellular matrix refers to an ECM which has been produced, secreted, supplied or deposited by one or more PDL cells.

[000118] As used herein, "porosity" means the ratio of the volume of interstices of a material to a volume of a mass of the material.

[000119] As used herein, the term "cell" refers to a membrane-bound biological unit capable of replication or division.

[000120] As used herein, "stem cell" e.g., a mesenchymal stem cell, means an unspecialized cell that has the potential to develop into many different cell types in the body, such as mesenchymal osteoprogenitor cells, osteoblasts, osteocytes, osteoclasts, chondrocytes, and chondrocyte progenitor cells. Preferably the cells are from a compatible human donor. More preferably, the cells are from the patient (i.e., autologous cells).

[000121] As used herein, "osteoblast" shall mean a bone-forming cell which forms an osseous matrix in which it becomes enclosed as an osteocyte. It may be derived from mesenchymal stem or progenitor cells with osteogenic potential (e.g. osteoprogenitor cells). The term may also be used broadly to encompass osteoblast-like, and related, cells, such as osteocytes and osteoclasts. An "osteoblast-like cell" means a cell that shares certain characteristics with an osteoblast (such as expression of certain proteins unique to bones), but is not an osteoblast. "Osteoblast-like cells" include preosteoblasts and osteoprogenitor cells. Preferably the cells are from a compatible human donor. More preferably, the cells are from the patient (i.e., autologous cells).

[000122] As used herein, "periodontal ligament cell" shall mean a cell that is isolated from the periodontal ligament. Such a periodontal ligament cell can include differentiated cells and periodontal ligament stem cells.

[000123] As used herein, a "biocompatible" material is a synthetic or natural material used to replace part of a living system or to function in intimate contact with living tissue. Biocompatible materials are intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body. The biocompatible material has the ability to perform with an appropriate host response in a specific application and preferably does not have toxic or injurious effects on biological systems.

[000124] As used herein, a "biodegradable" graft is a graft that once implanted into a host, will begin to degrade. The implantable grafts of the present invention may be used in open surgical procedures as may be required and determined by a dental surgeon. Preferably, the implantable

graft is biomimetic and biodegradable. The rate of biodegradation may be engineered into the scaffolds based on the polymers used, the ratio of copolymers used, and other parameters well known to those of skill in the art. Moreover, in certain embodiments of the present invention, the rate of biodegradation of each phase may be separately engineered according to the needs of the particular surgery to be performed.

[000125] As used herein, the terms "treatment" or "treating" mean: (1) improving or stabilizing the subject's condition or disease or (2) preventing or relieving the development or worsening of symptoms associated with the subject's condition or disease.

[000126] As used herein, the terms "prevent," "preventing," "prevention," and the like refer to wholly or partially reducing the probability of acquiring an infection or re-infection in a subject, who does not have, but is at risk of or susceptible to infection.

[000127] As used herein, the terms "subject" and "patient" are used interchangeably. They refer to a human or another mammal (e.g., mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate) that can be afflicted with or is susceptible to a disease or disorder but may or may not have the disease or disorder. In certain embodiments, the subject is a human being.

[000128] As used herein, the term "agent" means any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide or fragment thereof.

[000129] Where the terms "comprise", "comprises", "comprised" or "comprising" are used in this specification (including the claims) they are to be interpreted as specifying the presence of the stated features, integers, steps or components, but not precluding the presence of one or more other features, integers, steps or components, or group thereof. For example, a graft comprising two scaffolds may contain two scaffolds only (i.e., “consist of” two scaffolds), or alternatively may contain 3, 4 or 5 scaffolds.

[000130] A reference herein to a patent document or other subject matter which is given as prior art is not to be taken as an admission that the document or subject matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

[000131] **Biphasic periodontal tissue grafts**

[000132] The present invention is directed towards biphasic periodontal tissue grafts wherein each phase of the tissue graft corresponds to either the hard or soft tissue of the periodontium.

[000133] The goal of periodontal therapy is the regeneration of the original architecture and function of the periodontal complex, which involves the formation of new cementum on the tooth root, along with new periodontal attachment between newly formed bone and cementum. However, on account of the complex structure of the periodontium, which consists of soft tissue including the gingiva, periodontal ligament, and hard tissue including bone and cementum, which compete during the regenerative process, effective treatments have been difficult to develop.

[000134] A periodontal tissue graft should contain a scaffold material that is sufficiently robust to not only resist distortion as a result of the introduction of cells into the scaffold, but also the wound contraction forces which will be invoked during tissue healing *in vivo*. The scaffold architecture preferably allows for initial cell attachment and subsequent migration into and through the scaffold, as well as mass transfer of nutrients and metabolites, while providing sufficient space for development and later remodeling of organized tissue. The porosity and internal space within a degradable scaffold generally increases with time, allowing increased space for tissue maturation and remodeling.

[000135] Accordingly, the present invention provides a decellularized biphasic periodontal tissue graft comprising a first polymeric scaffold comprising a plurality of first polymer fibers and a second polymeric scaffold comprising a plurality of second polymer fibers.The first and second polymeric scaffolds are adjoined/interconnected, meaning that there is at least some degree of interaction between the scaffolds keeping them in contact and in proximity. The first polymer fibers may be arranged such that said first polymeric scaffold has a porous structure with an average first pore size. Similarly, the second polymer fibers may be arranged such that the second polymeric scaffold has a porous structure with an average second pore size. The average first and average second pore size may be the same or different.The first and/or second polymer fibers may be coated with an extracellular matrix. For example, the first polymer fibers may be coated with an osteoblast-derived extracellular matrix and/or second polymer fibers may be coated with a periodontal ligament cell-derived extracellular matrix.

[000136] In one embodiment, the average diameter of the polymer fibers of the first polymeric scaffold is from about 50 μm to about 500μm, about 50 μm to about 400μm, about 150 μm to about 500μm, or about 200 μm to about 400μm. In another embodiment, the average diameter of the polymer fibers of the first polymeric scaffold is about 50, 100, 150, 200, 250, 300, 350, 400, 450, or 500 μm. In another embodiment, the average diameter of the polymer fibers of the first polymeric scaffold is from about 500nm to about 50μm. In another embodiment, the average diameter of the polymer fibers may be about 500nm, 600nm, 700nm, 800nm, 900nm or 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45 or 50μm. In another embodiment, the average diameter of the polymer fibers may be between about 1μm and 40μm, 5μm and 40μm,10μm and 40μm,10μm and 30μm, or about 20μm and 30μm.

[000137] In another embodiment of the present invention, the polymer fibers of the first polymeric scaffold are arranged so as to provide a porous scaffold wherein the average pore diameter of the first polymeric scaffold is from about 10μm to about 1000 μm, about 50μm to about 800 μm, about 100μm to about 750 μm, about 200μm to about 600 μm, or about 300μm to about 500 μm. In another embodiment, the average pore diameter may be about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000μm. In another embodiment, the average pore diameter of the first polymeric scaffold is from about 50 μm to about 500μm. In another embodiment, the pore diameter may be about 50, 100, 150, 200, 250, 300, 350, 400, 450, or 500 μm.

[000138] In one embodiment, the average diameter of the polymer fibers of the second polymeric scaffold is from about 500nm to about 50μm. In another embodiment, the average diameter of the polymer fibers may be about 500nm, 600nm, 700nm, 800nm, 900nm or 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45 or 50μm.

[000139] In another embodiment the of the present invention, the polymer fibers of the second polymeric scaffold are arranged so as to provide a porous scaffold wherein the average pore diameter of the second polymeric scaffold is from about 10μm to about 500μm, about 100μm to about 400μm, about 200μm to about 500μm, about 10μm to about 500μm, or about 10μm to about 500μm. In another embodiment, the pore diameter fibers may be about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 or 1000μm. In another embodiment, the average pore diameter of the second

polymeric scaffold is from about 1 μm to about 10μm. In another embodiment, the pore diameter is from about 1, 2, 3, 4, 5, 6, 8, 9, 10μm.

[000140] In another embodiment of the invention, the first scaffold comprises a fixed deposition molded polymer or a melt-electrospun polymer and said second scaffold comprises a melt-electrospun polymer or a solution-electrospun polymer.

[000141] The use of tissue engineered grafts populated with a patient's own cells represents a promising avenue. However, there are underlying limitations in reaching clinical practice, such as the reliance on the patient's own cells, as well as the need for cell culture facilities and associated technical expertise.

[000142] Accordingly, the present invention provides a biphasic tissue graft wherein the phases are polymeric scaffolds which are prepopulated with cells specific to each phase and subsequently decellularized such that cells and cellular components are removed but an extracellular matrix (ECM) deposited by the cells of each phase is retained. The retention of such ECM facilitates an ordered population of the graft by host cells and enhanced repair or regeneration of the periodontium.

[000143] In one embodiment of the present invention the polymers of the first polymeric scaffold are coated with extracellular matrix which is derived from osteoblasts and/or osteoblast-like cells. The osteoblasts and/or osteoblast like cells may be directly isolated from an individual (e.g., an intended graft recipient or a subject that is not the intended graft recipient) or may result from the directed differentiation of stem or progenitor cells.

[000144] In one embodiment of the present invention the polymers of the first polymeric scaffold are coated with extracellular matrix which is derived from periodontal ligament cells. The periodontal ligament cells may be directly isolated from an individual (e.g., an intended graft recipient or a subject that is not the intended graft recipient) or may result from the directed differentiation of stem or progenitor cells.

[000145] In these embodiments, the tissue grafts may further comprise osteoblasts, osteoblast-like cells, periodontal ligament cells, stem cells, progenitor cells or combinations thereof. In this aspect, osteoblasts, osteoblast-like cells, stem cells, progenitor cells or combinations thereof are disposed on at least a portion of the first phase. In a further aspect, periodontal ligament cells,

stem cells, progenitor cells or combinations thereof are disposed on at least a portion of the second phase. In yet a further aspect, osteoblasts are disposed on the first phase and periodontal ligament cells are disposed on the second phase.

[000146] The tissue grafts of the present invention may be submitted to one or more coating processes prior to being cultured with certain cells, in accordance with the invention. In one embodiment, a calcium phosphate coating process by successive immersion into specific reagents and solutions is applied to the grafts. Without limitation to a particular mode of action, the inventors have found that CaP coating increases the roughness and surface area of the PCL membrane which results in an increased loading efficiency and uniform distribution of another agent to be loaded onto the scaffold. In a further embodiment, the additional agent is an anti-inflammatory/antibiotic agent.

[000147] In another aspect of these embodiments, at least one of the phases further comprises a bioactive agent selected from an anti-infective, an extracellular matrix component, an antibiotic, bisphosphonate, a hormone, an analgesic, an anti-inflammatory agent, a growth factor, an angiogenic factor, a chemotherapeutic agent, an anti-rejection agent, an RGD peptide, and combinations thereof.

[000148] In a preferred embodiment, the tissue graft according the first and/or second polymeric scaffold is coated with one or more anti-inflammatory agents and/or one or more antibacterial agents.

[000149] In one embodiment, an external surface of said first polymeric scaffold is coated with a membrane comprising said one or more anti-inflammatory agents and/or one or more antibacterial agents. In another embodiment, the membrane is a non-porous occlusive scaffold. The membrane may coat all or only a portion of the external surface of the first polymeric scaffold. The membrane may also contact and/or coat a portion of an external surface of the second polymeric scaffold in a manner that does not prevent attachment of the second polymeric scaffold to the tooth surface.

[000150] Classes of antibiotic compositions that may be useful in the tissue grafts and the methods of the present disclosure for producing thetissue grafts include aminoglycosides exemplified by tobramycin, gentamicin, neomycin, streptomycin, and the like; azoles exemplified by fluconazole, itraconazole, and the like; β-lactam antibiotics exemplified by

penams, cephems, carbapenems, monobactams, β-lactamase inhibitors, and the like; cephalosporins exemplified by cefacetrile, cefadroxyl, cephalexin, cephazolin, cefproxil, cefbuperazone, and the like; chloramphenicol; clindamycin; fusidic acid; glycopeptides exemplified by vancomycin, teicoplanin, ramoplanin, and the like; macrolides exemplified by azithromycin, clarithromycin, dirithromysin, erythromycin, spiramycin, tylosin, and the like; metronidazole; mupirocin; penicillins exemplified by benzylpenicillin, procaine benzylpenicillin, benzathinebenzylpenicillin, phenoxymethylpenicillin, and the like; polyenes exemplified by amphotericin B, nystatin, natamycin, and the like; quinolones exemplified by ciprofloxacin, ofloxacin, danofloxacin, and the like; rifamycins exemplified by rifampicin, rifabutin, rifapentine, rifaximin, and the like; sufonamides exemplified by sulfacetamine, sulfadoxine, and the like; tetracyclines exemplified by doxycycline, minocycline, tigecycline, and the like; and trimethoprim, among others.

[000151] In a preferred embodiment the one or more antibiotic agents are selected from the group consisting of macrolides, β-Lactams, metronidazole and tetracyclines.

[000152] In a preferred embodiment, the antibiotic agent is hydrophobic in nature.

[000153] In another embodiment, the one or more antibiotic agent is selected from, doxycycline, amoxicillin and metronidazole.

[000154] In a preferred embodiment, one or more antibiotic agents are selected which comprise both antibiotic and anti-inflammatory activities.

[000155] In a more preferred embodiment the antibiotic is a macrolide. In a particularly preferred embodiment, the agent is azithromycin. In another embodiment of the invention, the azithromycin is present in an amount selected from any one of about 10μg to about 100μg, about 100μg to about 250μg, about 250μg to about 500μg, about 500μg to about 750μg, about 750μg to about 1000μg, about 1000μg to about 5000μg per graft. In another embodiment the azithromycin is present in an amount of about 50μg to about 1000μg per graft. In another embodiment the azithromycin is present in an amount of about 250μg per graft. In another embodiment the azithromycin is present in an amount of about 100μg per graft.

[000156] In another embodiment, the azithromycin is present in an amount selected from any one of about 0.7μg/mm^3 to about 3μg/mm^3, about 3μg/mm^3 to about 10μg/mm^3, about 10μg/mm^3 to about 20μg/mm^3, about 20μg to about 50μg/mm^3, about 50μg/mm^3 to about 100μg/mm^3, about 100μg/mm^3 to about 200μg, about 200μg/mm^3 to about 300μg/mm^3, about 300μg/mm^3 to about 400μg/mm^3, about 400μg/mm^3 to about 500μg/mm^3 . Preferably, the azithromycin is present in an amount of about 3μg/mm^3 to about 100μg/mm^3. In another embodiment, azithromycin is present in an amount of about 20μg/mm^3. In another embodiment, azithromycin is present in an amount of about 10μg/mm^3. In yet another embodiment, azithromycin is present in an amount of about 5μg /mm^3.

[000157] In a particular embodiment, a combination of antibiotics may be employed. This may include a combination of azithromycin and another hydrophobic antibiotic. Alternatively, a combination comprising azithromycin and a bacteriostatic antibody may be employed.

[000158] In a preferred embodiment the one or more anti-inflammatory agents are selected from the group consisting of resolvins, protectins, lipoxins and other anti-inflammatory eicosanoids.

[000159] The tissue grafts of the present invention may comprise natural and non-natural polymers which may be biodegradable or non-biodegradable. For example, polymers which may be employed in the present invention include biodegradable synthetic polymers including: Poly esters, exemplified by poly(glycolic acid) (PGA), poly(lactic acid) (PLA), Poly(glycoloc-co-lactic acid) (PLGA), Poly(dioxananone) Poly(caprolactone) (PCL), Poly(3-hydroxybutyrte), Poly(3-hydroxyvalerate), Poly(valerolactone), Poly(tartonic acid), Poly(β-malonic acid), Poly(propylene fumarates) (PPF), and the like; Poly anhydrides exemplified by poly[1,6-bis(carboxyphenoxy)hexane] and the like; Poly anhydrides –co-imides, exemplified by poly-[trimellitylimidoglycine-co-bis(carbox-yphenoxy) hexane], poly[pyromellitylimidoalanine-co-1,6-bis(carboph-enoxy)-hexane], and the like; Poly carbonates, exemplified by Tyrosine-derived polycarbonate, and the like; Poly orthoesters; Poly urethanes exemplified by poly(tetramethylene oxide) (PTMO), poly urethanes based on lysine diidocyanate and poly(glycolide-co-γ polycaprolactone) and the like; Poly phosphazenes exemplified by Ethylglycinate poly phosphazenes and the like; Poly(glycerol sebacate) and Poly ethylene glycol; Biodegradable natural polymers selected from Extracellular Matrix components exemplified by Collagen, Fibronectin, Glycosaminoglycans (GAGs), Fibrin and the like; Poly saccharides exemplified by Starch, Cellulose, Arabinogalactan (larch gum), Alginic acid, Agar,

Carrageenan, Chitin (chitosan, carboxymethyl chitin), Hyaluronic acid, Dextran, Gellan gum, Pullan and the like; Protein derived polymers including silk proteins (Silk fibers); Poly esters exemplified by poly hydroxyalkanoates (PHA), poly(hydroxybutyrate), PHB, poly(hydroxybutyrate-co-valerate), PHBV and the like.

[000160] Non degradable polymers which may be employed in the present invention include: Poly ethylene, Poly propylene, Poly tetrafluoroethylene Poly acrylate exemplified by Poly (2 hydroxyethly methacrylate), Poly (2 hydroxyethly methacrylate)/terahydrofurfuryl methacrylate, Poly methyl methacrylate and the like, Poly dimethylsiloxane, Poly ether urethanes, Poly ethylene terphathalate, Poly sulfone, Poly ethyleneoxide, Poly vinyl alcohol, Poly (N-isopropylacrilamide) and composites such as Poly (N-isopropylacrilamide- co acrylic acid).

[000161] The biomaterials to be used in accordance with the invention are generally biocompatible and biodegradable polymers, which may be of natural and/or synthetic origin. For example, the biomaterials can be selected from a group of synthetic polymers consisting of but not limited to aliphatic polyesters, poly(amino acids), modified proteins, polydepsipeptides, copoly(ether-esters), polyurethanes, polyalkylenes oxalates, polyamides, poly(iminocarbonates), polyorthoesters, polyoxaesters, polyamidoesters, poly(ε-caprolactone)s, polyanhydrides, polyarylates, polyphosphazenes, polyhydroxyalkanoates, polysaccharides, modified polysaccharides, polycarbonates, polytyrosinecarbonates, polyorthocarbonates, poly(trimethylene carbonate), poly(phosphoester)s, polyglycolide, polylactides, polyhydroxybutyrates, polyhydroxyvalerates, polydioxanones, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(maleic anhydride), polyvinylalcohol, polyesteramides, polycyanoacrylates, polyfumarates, poly(ethylene glycol), polyoxaesters containing amine groups, poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(dioxanone)s, poly(alkylene alkylate)s, biopolymers, collagen, silk, chitosan, alginate, and a blend or copolymers of two or more of the preceding polymers.

[000162] A particular polymer or combination of polymers for use in the tissue grafts of the present invention may be selected according to a desired rate of resorption of one or more phases of the graft.

[000163] In a preferred embodiment, the biphasic tissue graft comprises polycaprolactone.

[000164] Through a process of seeding, culturing and subsequent decellularization of a polymeric scaffold of biphasic tissue graft of the present invention, the surfaces of the polymeric fibres may be pre-coated with extracellular matrix components. The extracellular matrix components include but are not limited to collagens, elastins, laminins, glycosaminoglycans, proteoglycans, antimicrobials, chemoattractants, cytokines, and growth factors. These extracellular matrix components are utilized to facilitate the initial attachment of the regenerative cells on the scaffold surfaces following in vitro seeding or more particularly in vivo implantation as part of a regenerative therapeutic procedure utilizing the biphasic tissue grafts. In a particular embodiment of the invention, the polypeptide growth factors, cytokines and/or chemoattractant proteins are included in the bi-phasic tissue grafts and are involved in stimulating the chemotaxis, migration and proliferation of cells including regenerative stem and progenitor cells from the adjacent healthy tissues into the scaffolds, inducing the differentiation and supporting the maintenance of viability and functionality of the cells, thereby enhancing the formation of new regenerated tissues.

[000165] In a particular embodiment, the osteoblast-, and periodontal ligament cell-derived extracellular matrices include one or more collagens, proteoglycans, glycoproteins, fibronectin, laminin, adhesive glycoproteins, glycosaminoglycans (GAG), chemoattractants, cytokines, growth factors, other non-collagenous proteins, osteocalcin, osteopontin, bone sialoprotein, periostin, sclerostin, cementum attachment protein, and/or any molecule that may be found within the native bone, periodontal ligament and cementum extracellular matrix.Type I collagen and fibronectin are two of the predominant proteins present in the native periodontal ligament tissue. Amongst the various growth factors present, are basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).

[000166] In a particular embodiment, the extracellular matrix components comprise Type I collagen and fibronectin, bFGF, VEGF and HGF.

[000167] **Methods of manufacturing a decellularized biphasic periodontal tissue graft**

[000168] The present invention also provides methods for the production of decellularized biphasic periodontal tissue grafts.

[000169] According to one aspect the present invention provides a method of producing a decellularized biphasic periodontal tissue graft comprising laying a plurality of first polymer

fibers on a first surface to provide a first polymeric scaffold having a porous structure with an average first pore size, and, laying a plurality of second polymer fibers on a second surface to provide a second polymeric scaffold having a porous structure with an average second pore size. The first and second surfaces may be the same or different surfaces. In one embodiment, the first polymer fibers form the second surface. In another embodiment the second polymer fibers form the first surface.The first and said second polymeric scaffolds may be adjoined/interconnected to form a biphasic structure.The first and/or said second polymeric scaffolds may be seeded with one or more cell types or mixtures thereof. The seeding may occur before, during, or after adjoining/interconnecting the first and second polymeric scaffolds. In one embodiment the first polymeric scaffold may be seeded with osteoblasts and the second polymeric scaffold may be seeded with one or more layers of a periodontal ligament cell layer. Each seeded scaffold may be cultured in a suitable tissue culture medium.After culturing, biphasic tissue graft may be decellularized.

[000170] In one embodiment, a suitable first polymeric scaffold comprising a plurality of first polymer fibers, and having a porous structure with an average first pore size, may be provided in a pre-made form. For example, a fused deposition Modeling scaffold which is commercially available (as described in the examples herein) may be used as a first polymeric scaffold.

[000171] In a preferred embodiment, the laying of a plurality of polymer fibers is performed using an electrospinning technique. In a preferred embodiment, the laying of a plurality of first polymer fibers to provide a first polymeric scaffold occurs via a melt-electrospinning technique. In another preferred embodiment, the laying of a plurality of second polymer fibers to provide a second polymeric scaffold occurs via a melt-electrospinning techniqueor a solution-electrospinning technique.

[000172] According to a melt-electrospinning technique, by way of example only, molten polymer is electrospun at a preferred temperature and at a preferred rate and with a collector place at a preferred distance from the source of the polymer and for a preferred time so as to produce a polymer scaffold having preferred dimensions such as thickness and polymer fiber diameter and arrangement.

[000173] According to one embodiment, laying a plurality of first polymer fibers or a plurality of second polymer fibers is performed by loading polycaprolactone (PCL) pellets into a (e.g., 2

mL syringe) and electrospinning at a suitable temperature and feed rate (e.g., 80°C at 20 μL/h, at 7 kV and at a 4 cm tip to collector distance). Scaffolds of suitable diameter (e.g., 8 mm) may be produced by electrospinning the molten PCL for suitable periods (e.g., 4 minutes) onto a suitable surface (e.g.,aluminum foil-covered glass slides) placed over the collector.

[000174] Similarly, and by way of example only, a solution electrospinning technique can be performed according to a process wherein a polymer is first dissolved in an appropriate solvent or mixture thereof to create a polymer solution. The polymer solution is then electrospun at a preferred rate and with a collector place at a preferred distance from the source of the polymer solution and for a preferred time so as to produce a polymer scaffold having preferred dimensions such as thickness and polymer fiber diameter and arrangement.

[000175] According to one embodiment, laying a plurality of second polymer fibers may be performed by loading mixture of polymer and chloroform and dimethylformamide (9/1 vol/vol) at a concentration of 15% wt/vol. The polymer solution may be loaded into a 10 mL syringe and electrospun at a feed rate of 2 mL/h, at 10 kV and at a 20 cm tip to collector distance for 30 min. A scaffold prepared according to this method was shown to have a thickness of approximately 300-400 μm and a fiber diameter of 3 μm, a small pore size from 5 to 10 μm.

[000176] First and second polymeric scaffolds prepared as outlined above may be adjoined/interconnected to one another by any suitable means known to the skilled addressee. According to one preferred embodiment a surface of the first polymeric scaffold is placed in abutting arrangement with the second polymeric scaffold. In one embodiment of the invention, the method involves placing the first polymeric scaffold in close proximity to a heat source (e.g. a hot plate) so as to partially melt a portion of the first scaffold and then press-fitting the first scaffold onto the second scaffold. In one embodiment, the first scaffold is placed, for example, at a distance of about 1 cm from a hot plate heated to about 300°C for about 4 seconds, and then quickly press-fitted for about 10 seconds onto the second scaffold.

[000177] After assembly of first and second polymeric scaffolds of the biphasic tissue graft, the scaffold may be seeded with one or more cells. Subsequently, the scaffold may be cultured under appropriate conditions and in a tissue culture medium that will promote the attachment, migration and proliferation of the cells seeded in the scaffold, such that deposition of a sufficient amount of extracellular matrix is achieved. Such amount may be readily determined by one of

skill in the art, such as by a colorimetric biochemical assay or immunoassay directed towards a particular component of the ECM. Seeding and culturing of the first and/or second polymeric scaffold may occur before, during or after adjoining/interconnecting the first and first and/or polymeric scaffolds.

[000178] In a preferred embodiment, the first scaffold is seeded with one or moreosteoblasts, osteoblast-like cells, stem cells, progenitor cells or combinations thereof are. The osteoblasts or osteoblast like cells may be directly isolated from an individual (e.g., the intended recipient or another different individual), or may result from the directed differentiation of stem or progenitor cells (which may also be obtained from the intended recipient or another different individual). Accordingly, the cells may be isolated directly from a subject into which a biphasic tissue graft according to the present invention is to be implanted or they may be an independent donor.

[000179] In a preferred embodiment the first polymeric scaffold is seeded with one or more osteoblasts (e.g., 200,000 cells in 40 μL of media) and allowed to adhere (e.g., 4 hours at 37°C in a 5% CO2 atmosphere) before the well is filled with media. The biphasic tissue graft maythen be turned upside down with the second polymeric scaffold facing upwards in order to minimize cell infiltration into the second scaffold. The graft may then be cultured (e.g., for 6 weeks) in an osteogenic medium (e.g., media supplemented with 50 μg/mL ascorbate-2-phosphate, 10 mM β-glycerophosphate, 0.1 μM dexamethasone). The biphasic tissue grafts may beentirely covered by the medium. Sufficient osteoblast proliferation and activity may be monitored by routine assays throughout the culture period.

[000180] In another preferred embodiment, the second scaffold may be seeded with one or more periodontal ligament cells, stem cells, progenitor cells or combinations thereof. The PDL cells may be directly isolated from an individual or may result from the directed differentiation of stem or progenitor cells. The cells may be isolated directly from a subject into which a biphasic tissue graft according to the present invention is to be implanted or they may be an independent donor.

[000181] In a preferred embodiment the second polymeric scaffold may be seeded with one or more PDL cell sheets.

[000182] By way of non-limiting example only, PDL cell sheets may be obtained according to the following procedure. A culture of cells may be isolated from periodontal tissues obtained from the middle third of the roots, and explanted into a suitable vessel(e.g., 25 cm^2 flasks). The cells may be subsequently grown and propagated (e.g., in 175 cm^2 flasks with Dulbecco's Modification of Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), Penicillin (50units/ml) and Streptomycin (50µg/ml)).hPDL cells may be seeded in cell culture wells at an appropriate seeding density (e.g., $5x10^4$ cells/well in media supplemented with ascorbic acid (1000 µg/ml)). For the first 48 hours or thereabouts, the ascorbic acid concentration may be up to ten fold greater than the standard concentration in order to enhance early extracellular matrix formation. The cells may be grown in culture for a suitable time period (e.g., 19 days in media supplemented with ascorbic acid (100 µg/ml), which may be changed every 48 hours).At the end of the culture period (e.g. 21 days), the borders of the cell sheet can be gently detached from the base of the well and folded and pulled towards the edges of the second scaffold using sterile fine curved tweezers. To promote attachment of the PDL sheets to the second scaffold, samples may befurther incubated in culture media (e.g., for 24 hours).

[000183] As described herein, the methods of the present invention allow for the production of a bi-phasic periodontal tissue graft which is decellularized after being seeded with cells and cultured for a time so as to promote cell attachment, migration and proliferation within the graft and thereby deposition of extracellular matrix. The decellularization of the matrix may be performed according to any suitable method known to the skilled addressee. However, it is preferred that a sufficient amount of extracellular matrix deposition on the scaffolds of the biphasic graft be retained following the decellularization.

[000184] Accordingly, in a preferred embodiment of the invention a decellularization process comprises perfusing the tissue graft with a solution of NH_4OH solution and Triton X-100, followed by perfusing the graft with a DNase I solution, followed by perfusing the graft with sterile water. In another preferred embodiment each perfusion step is performed for about 30 minutes to 2 hours. In another preferred embodiment the perfusion of the solutions in the graft is bi-directional.

[000185] As outlined herein, the inventors have identified that coating the polymeric scaffolds with Calcium Phosphate (CaP)enables enhance mineralization of the scaffolds when seeded with

cells. Furthermore the inventors have surprisingly discovered that the CaP facilitates the coating of the scaffolds with additional agents such as azithromycin.

[000186] Accordingly in a preferred embodiment, a method for producing a biphasic periodontal tissue graft according to the invention further comprises a step of coating the a scaffold of the graft with CaP. The coating of the polymeric scaffold is preferably performed on the first polymeric scaffold corresponding to a bone compartment. The scaffolds may be coated with CaP according methods known to those skilled in the art. In a preferred embodiment the coating of a scaffold of the biphasic grafts of the invention is performed according to the procedure described in Example 2.

[000187] According to another aspect, the invention relates to a method of producing a decellularized biphasic periodontal tissue graft further comprising the step of coating the first and/or second scaffold with an anti-inflammatory and/or antibacterial agent prior to seeding the first scaffold (e.g., with osteoblasts).

[000188] This feature enables the local delivery of therapeutic and/or antibacterial agents which may be released from the grafts. The inventors have surprisingly discovered that coating the scaffolds with an anti-inflammatory/antibacterial agent enables the controlled and sustained release of the agent which will have significant effects in reparative and regenerative processes.

[000189] According to a preferred embodiment, the method of producing a decellularized biphasic periodontal tissue graft further comprises the step of coating the first and/or second scaffold with one or more anti-inflammatory agents and/or one or more antibacterial agents.

[000190] In one embodiment, the coating step comprises applying to an external surface of said first polymeric scaffold a membrane comprising said one or more anti-inflammatory agents and/or one or more antibacterial agents. In another embodiment, the membrane is a non-porous occlusive scaffold. The membrane may be applied such that all or only a portion of the external surface of the first polymeric scaffold is coated. The membrane may also contact and /or coat a portion of an external surface of the second polymeric scaffold in a manner that does not prevent attachment of the second polymeric scaffold to the tooth surface.

[000191] Classes of antibiotic compositions that may be useful in the tissue grafts and the methods of the present disclosure for producing the tissue grafts include aminoglycosides

exemplified by tobramycin, gentamicin, neomycin, streptomycin, and the like; azoles exemplified by fluconazole, itraconazole, and the like; β-lactam antibiotics exemplified by penams, cephems, carbapenems, monobactams, β-lactamase inhibitors, and the like; cephalosporins exemplified by cefacetrile, cefadroxyl, cephalexin, cephazolin, cefproxil, cefbuperazone, and the like; chloramphenicol; clindamycin; fusidic acid; glycopeptides exemplified by vancomycin, teicoplanin, ramoplanin, and the like; macrolides exemplified by azithromycin, clarithromycin, dirithromysin, erythromycin, spiramycin, tylosin, and the like; metronidazole; mupirocin; penicillins exemplified by benzylpenicillin, procaine benzylpenicillin, benzathinebenzylpenicillin, phenoxymethylpenicillin, and the like; polyenes exemplified by amphotericin B, nystatin, natamycin, and the like; quinolones exemplified by ciprofloxacin, ofloxacin, danofloxacin, and the like; rifamycins exemplified by rifampicin, rifabutin, rifapentine, rifaximin, and the like; sufonamides exemplified by sulfacetamine, sulfadoxine, and the like; tetracyclines exemplified by doxycycline, minocycline, tigecycline, and the like; and trimethoprim, among others.

[000192] In a preferred embodiment the one or more antibiotic agents are selected from the group consisting of macrolides, β-Lactams and tetracyclines.

[000193] In a preferred embodiment, the antibiotic agent is hydrophobic in nature.

[000194] In another embodiment, the one or more antibiotic agent is selected from, doxycycline, amoxicillin and metronidazole.

[000195] In a preferred embodiment, one or more antibiotic agents are selected which comprise antibiotic and anti-inflammatory activities.

[000196] The amount of antibiotic to be loaded onto the grafts may be readily determined by the skilled addressee. For example the amount of antibiotic to be utilized may be determined according to the severity of an infection or potential infection.

[000197] In a more preferred embodiment the antibiotic is a macrolide. In a particularly preferred embodiment, the agent is azithromycin. In another embodiment of the invention, the azithromycin is present in an amount selected from any one of about 10μg per graft to about 100μg per graft, about 100μg per graft to about 250μg per graft, about 250μg per graft to about 500μg per graft, about 500μg per graft to about 750μg per graft, about 750μg per graft to about

1000μg per graft, about 1000μg per graft to about 5000μg per graft. In another embodiment the azithromycin is present in an amount of about 50μg to about 1000μg per graft. In another embodiment the azithromycin is present in an amount of about 250μg per graft. In another embodiment the azithromycin is present in an amount of about 100μg per graft.

[000198] In another embodiment, the azithromycin is present in an amount selected from any one of about 0.7μg/mm^3 to about 3μg/mm^3, 3μg/mm^3 to about 10μg/mm^3, about 10μg/mm^3 to about 20μg/mm^3, about 20μg to about 50μg/mm^3, about 50μg/mm^3 to about 100μg/mm^3, about 100μg/mm^3 to about 200μg, about 200 μg/mm^3 to about 300 μg/mm^3, about 300 μg/mm^3 to about 400 μg/mm^3, about 400 μg/mm^3 to about 500 μg/mm^3 . Preferably, the azithromycin is present in an amount of about 3μg/mm^3 to about 100 μg/mm^3. In another embodiment, azithromycin is present in an amount of about 20 μg/mm^3. In another embodiment, azithromycin is present in an amount of about 10 μg/mm^3. In yet another embodiment, azithromycin is present in an amount of about 5 μg /mm^3.

[000199] In a particular embodiment, a combination of antibiotics may be employed. This may include a combination of azithromycin and another hydrophobic antibiotic. Alternatively, a combination comprising azithromycin and a bacteriostatic antibody may be employed.

[000200] In a preferred embodiment the one or more anti-inflammatory agents are selected from the group consisting of resolvins, protectins, lipoxins and other anti-inflammatory eicosanoids.

[000201] In a further preferred embodiment, the scaffold is coated with by azithromycin the steps of dissolving an amount of azithromycin in ethanol and applying to the tissue graft; incubating the graft for a suitable time period; evaporating the ethanol at room temperature and washing the grafts after the evaporation of ethanol. Typically, about 80-95% percent of the total amount of azithromycin present in the ethanol solution is encapsulated on the graft. In a preferred embodiment, at least 90% of azithromycin is encapsulated on the graft.

[000202] According to another embodiment of the invention, the methods for the production of a personalized biphasic periodontal tissue graft comprises 3D imaging of the periodontium of the subject and tissue printing.

[000203] The 3D scanning, modeling and printing process is a computerized state-of-the-art technology and materials application for the design, manufacture and production of personalized, custom-fitted biphasic tissue grafts according to the present invention. The scanning process includes but is not limited to 3D portable, handheld, and fixed unit scanners, digital cameras, smart phones, smart pads and other imaging technologies to record multiple perspective images of periodontal tissue to be regenerated or repaired, such as a periodontal defect or injury, and surrounding tissue to create a 3D model design of the scanned area.

[000204] The composite image or 3D model design is typically uploaded to a computer with 3D modeling and design software. The resulting 3D model design of the periodontal tissue and surrounding tissue is edited and integrated into the graft design. The personalized custom-fitted design can enable a more physiologically and anatomically adapted tissue graft. (See, for example, Figure 22).

[000205] The final integrated 3D model design is uploaded to a 3D printer to manufacture a custom-fitted tissue graft according to the present invention. The 3D printing process includes but is not limited to selective laser sintering and fusion deposition 3D printing technologies. Selective laser sintering and fused deposition are additive processes that use the melting of fine powders or softening of a polymer to produce a 3D shape by adding material in layers. There is an array of materials available to produce the biphasic periodontal tissue graft of the present invention including but not limited to various polymers.

[000206] Sterilization

[000207] In another embodiment of the invention the tissue grafts hereinbefore described may be sterilized according to methods known and used by the skilled addressee. In one embodiment, the sterilization is via ethlylene oxide, UV irradiation or gamma irradiation.

[000208] Methods of Tissue Repair/Regeneration

[000209] According to another aspect, the present invention relates to the use of a decellularized biphasic periodontal tissue graft as defined herein or a decellularized biphasic periodontal tissue graft obtained by or obtainable by the methods of the invention defined herein, for the repair and/or regeneration of an intrabony, infrabony, suprabony and/or furcation or other periodontal defect,for the treatment of periodontal disease or degeneration, or for the regeneration of

periodontal tissue in a subject, by the implantation of such a graft. The implantation of the grafts according to the invention into a subject in need thereof may be easily performed by those of skill in the art. The tissue grafts according to the invention may be appropriately shaped or designed in order to facilitate the most optimal tissue repair and or regeneration.

[000210] In one embodiment a decellularized biphasic periodontal tissue graft as defined herein, or a decellularized biphasic periodontal tissue graft obtained by or obtainable by the methods of the invention defined herein, used in a method of tissue repair or regeneration may be administered alone.

[000211] In a one embodiment, the use of a decellularized biphasic periodontal tissue graft as defined herein for the repair and/or regeneration of an intrabony, infrabony, suprabony and/or furcation or other periodontal defect, for the treatment of periodontal disease or degeneration, or for the regeneration of periodontal tissue in a subject, involves the application of a membrane comprising an antibiotic and/or anti-inflammatory agent on an external surface (facing the gingival connective tissue) of the tissue graft at the time of implantation. The membrane may be positioned so as to coat all or only a portion of the external surface of the first polymeric scaffold. The membrane may also contact and/or coat a portion of an external surface of the second polymeric scaffold in a manner that does not prevent attachment of the second polymeric scaffold to the tooth surface.

[000212] **Combination therapy**

[000213] Optionally, the aforementioned grafts may be administered in combination with any other standard therapy for the treatment of a periodontal disease of defect where tissue repair or regeneration is required. If desired, agents of the invention may be administered alone or in combination with a conventional therapeutic useful for the treatment of a periodontal disease of defect. Such agent may be administered separately to the graft or in association with the graft. That is, the therapeutic agent may be coated on, impregnated within, attached or otherwise bonded to the graft.

[000214] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

Examples

Example 1. Generation of a biphasic periodontal tissue graft

[000215] The following example describes the generation of a biphasic tissue graft comprising wherein one phase corresponds to a bone compartment and another phase corresponds to a periodontal compartment.

[000216] **Materials and Methods**

[000217] Polycaprolactone (PCL medical grade 80 kDa) and PCL β-TCP (20% wt, β-Tricalcium Phosphate) Fused Deposition Modelling scaffolds (0/60/120, 100x100x2 mm3) were obtained from Osteopore (Singapore). Prior to use the FDM scaffolds were sectioned into 5x5x2 mm^3 pieces.

[000218] Electrospinning

[000219] PCL was electrospun using an in-house device. The polymer was first dissolved in a mixture of chloroform and dimethylformamide (9/1 vol/vol) at a concentration of 15% wt/vol. The polymer solution was loaded into a 10 mL syringe and electrospun at a feed rate of 2 mL/hr, at 10 kV and at a 20 cm tip to collector distance for 30 min.

[000220] Biphasic tissue graft fabrication:

[000221] The biphasic tissue graft consisted of a FDM component for the bone compartment and an electrospun mesh for the periodontal compartment. To assemble the biphasic tissue graft, the FDM component was placed 1 cm from a hot plate heated at 300 °C for 4 s and then quickly press-fitted for 10 s onto a piece of electrospun PCL (7x9x0.4 mm3). This heat treatment partially melted the first layer of the FDM component enabling it to strongly bind to the electrospun scaffold upon cooling and solidification.

[000222] Scaffold Morphology

[000223] Scanning electron microscopy (SEM) was performed to investigate cohesion between the two components. Tissue grafts were immersed in liquid nitrogen for 5-10 min and a sharp

razor blade was used to section the structures. The samples were gold coated for 3 min and observed with a FEI Quanta 200 Environmental SEM operating at 10 kV.

[000224] In vitro study

[000225] Cell isolation and culture

[000226] Osteoblasts

[000227] Ovine mandibular osteoblast explants were obtained from Merino sheep undergoing experimental surgery. Compact bone samples were collected under sterile conditions from the mandible under general anaesthesia with a trephine drill (5 mm diameter), minced, washed with phosphate buffered saline (PBS) and vortexed 5 times. Bone samples were then incubated with 10 mL of 0.25% trypsin/EDTA for 3 min at 37°C in a 5% CO2 atmosphere. After trypsin inactivation, samples were washed once again with PBS and transferred in basal culture media into 175 cm2 tissue culture flasks. Outgrowth of osteoblasts was observed after 5-7 days. Cells were expanded and used at the third passage (P3).

[000228] Periodontal ligament cells:

[000229] Periodontal ligament cells were also obtained from Merino sheep undergoing experimental surgery. Two incisors were first extracted and placed into a 50 mL tube containing DMEM with 2% penicillin/streptomycin and 4 µg/mL fungizone. The middle third of the periodontal ligament (PDL) was subsequently gently removed from the root surface with a scalpel and further sectioned into approximately 1x1 mm^2 pieces. The PDL tissues were placed into a 25 cm^2 culture flask which was left standing upright in an incubator at 37°C and 5% CO2 atmosphere for 30 min to allow tissue adhesion. After this incubation period, 3 mL of DMEM containing 10% FBS, 100 U/mL of penicillin/streptomycin and 0.1 µg/mL fungizone were added and the flask was carefully laid down in the incubator. The first media change occurred 4 days post extraction. After one week of culture, cells started migrating outwards from the PDL tissues and they generally reached confluence after 2-3 weeks of culture. The cells were passaged using 0.25% trypsin and further expanded until P3.

[000230] 2D culture

[000231] Osteoblasts and PDL cells were cultured separately in 24-well plates in order to assess their mineralization potential. Osteoblasts were seeded at 500 cells/cm^2 whereas the PDL cells were seeded at 250 cells/cm2 due to early cell sheet contraction at higher cell seeding densities. Both cell lines were cultured under basal or osteogenic induction media (50 μg/mL ascorbate-2-phosphate, 10 mM β-glycerophosphate and 0.1 μM dexamethasone) for 2 weeks. DNA content and ALP activity were measured in the same samples at days 7 and 14. The cells were also stained with alizarin Red S in order to assess the deposition of mineralized matrix.

[000232] DNA content and Alkaline phosphatase activity

[000233] ALP activity was measured in the cell culture media after a 24 hours release period. Briefly, samples were first rinsed in DMEM without phenol red three times and placed back in the incubator for precisely 24 hours. ALP activity was measured according to the SigmaFASTTM kit. 100 μL of p-Nitrophenyl phosphate in Tris-base buffer was added to 100 μL of the culture media in a 96-well plate. This was further incubated in for 24 hours. At the end of the second incubation period the plate was brought back to ambient temperature (20 °C) for 5 min and the absorbance was read at 405 nm using a plate reader (Benchmark PlusTM microplate spectrophotometer, BIO RAD).

[000234] For cellular DNA content analysis, the remaining media was removed from the wells and the samples frozen at -80 °C for at least 48 hours. The cell membrane and the extracellular matrix were disrupted in 300 μL of Proteinase K (Invitrogen) (Proteinase K/Phosphate Buffered EDTA (PBE) 0.5 mg/ml) at 37 °C overnight, then transferred into 1.5 mL Eppendorf tubes. The solution was thereafter diluted at a ratio of 1/50 in PBE and 100 μL of this diluted solution was aliquoted into black 96-well plates and 100 μL of PicoGreen (P11496, Invitrogen) working solution according to the manufacturer's instructions was added. After 5 min incubation in the dark the fluorescence (excitation 485 nm, emission 520 nm) was measured using a fluorescence plate reader. A standard curve was also constructed using known concentrations of λ DNA provided with the kit. The standard ranged from 10 ng/ml to 1 μg/mL λ DNA and was used to calculate the final DNA content of the sample.

[000235] Alizarin Red S staining

[000236] A 1% solution alizarin Red S was used to assess the deposition of mineralized matrix. The cells were rinsed twice in PBS and then fixed for 10 min in cold methanol. The cell sheet was then rinsed in ddH2O and 500 µL of alizarin solution was added for 10 min. The unfixed dye was removed by gently rinsing the stained cell sheet with ddH2O until a clear solution was obtained. The samples were air dried overnight and stored until use. The alizarin dye was extracted using 300 µL of a 50% acetic acid solution. The plates were placed onto a rocker for 10 min to allow for complete dissolution of the dye. The solution was then placed into 1.5 mL Eppendorf tubes and vortexed for 30 s. 150 µL of 4 M NaOH was added to bring the pH to 4.1. The tubes were then centrifuged at 10000 rpm for 10 min. Triplicates of 100 µL were placed into a 96-well plate and the absorbance was read at 405 nm.

[000237] 3D culture - Biphasic tissue graft seeding.

[000238] Osteoblasts (200,000 cells in 40 µL of media) were seeded onto the FDM component and allowed to adhere for 4 hours before the well was filled with media. Then, the biphasic tissue grafts were turned upside down in order to minimise cell infiltration into the electrospun component. Biphasic tissue grafts were further cultured for 3 weeks either in osteogenic media (50 µg/mL ascorbate-2-phosphate, 10 mM β-glycerophosphate, 0.1 µm dexamethasone) or in basal media. Osteoblast proliferation was measured by picogreen assay at 7, 14 and 21 days post seeding. Osteoblast morphology into the bone compartment was also imaged by SEM and confocal microscopy. For the SEM analysis the samples were fixed in a 2.5% glutaraldehyde solution, then dehydrated in a concentration gradient of ethanol and finally carbon coated. For confocal laser microscopy, the constructs were fixed in a 4% paraformaldehyde solution in PBS. The cell membrane was permeabilized for 5 min in a 0.2% triton X solution. After two rinses in PBS, the cells were stained for 45 min with a mixture of 0.8 U/mL TRITC-conjugated phalloidin and 5 µg/ml DAPI solution. The biphasic tissue grafts were rinsed another three times and imaged with a Leica SP5 microscope.

[000239] Harvesting of multiple cell sheets

[000240] PDL cells were seeded at 10,000 cells/cm2 in a 24-well plate. The cells were cultured in osteogenic media and as the cell sheet matured (after 7 days of culture), it started to contract and detach from the well. The PDL cell sheets were then harvested using the electrospun component of the biphasic tissue graft. The tissue graft was positioned in the centre of the well

and thereafter the cell sheet was folded over the edge of the electrospun mat using sterile needle sharp forceps. Once the cell sheet was harvested, the biphasic tissue graft was placed into a 6 well plate with the periodontal compartment facing upright. The bone compartment underneath was filled with media. 15 μL of media were added onto the top of the cell sheet to prevent the cells from drying out. The biphasic tissue grafts were placed back into the incubator and the cell sheet was hydrated every 10 min with media. The cell sheet was allowed to adhere onto the electrospun substrate for 30 min before another cell sheet was harvested. This procedure was repeated until 3 cell sheets were placed onto the biphasic tissue graft.

[000241] Cell viability was determined by staining the cell sheets with Fluorescein diacetate (FDA) at a dilution of 1/1000 from a stock solution (0.67 mg/mL dissolved in acetone, Invitrogen, USA) and propidium iodide (PI) at a dilution of 1/1000 from a stock solution (5 mg/mL dissolved in water, Invitrogen, USA). The cell sheet was then imaged with a Leica SP5 microscope.

[000242] In vivo study

[000243] Dentin slices and tissue graft assembling

[000244] One millimetre thick dentin slices were prepared from sheep teeth and adjusted to the size of the biphasic tissue grafts. The assembly of the biphasic tissue grafts onto dentin blocks was performed under sterile conditions and sutures were used to keep the biphasic tissue graft on top of the dentin slice. Thereafter the samples were immersed in media for 2 hours in order to allow for cell sheet adhesion onto the dentin surface. Notches on the dentin slides were created in order to prevent the sutures from sliding off ensuring high stability of the tissue graft.

[000245] Subcutaneous implantation

[000246] Animal ethics approval for the use of athymic nude rats in this experiment was granted by the Animal Ethics Committee of Griffith University. Five 8-week old male rats (Animal Resources Centre, Canning Vale, WA, Australia) were used. The animals were anaesthetized with isoflorane. Six small incisions were made longitudinally along the central line of the shaved dorsal area, approximately 2 cm apart, and subcutaneous pockets were made on each side of the incision with a pair of surgical scissors. Each individual pocket held one tissue graft. The incisions were closed with surgical sutures. The animals were sacrificed after eight weeks

and the implants were retrieved and fixed in 4% paralformaldehyde in PBS at pH 7.4 for further analysis.

[000247] Micro-CT analysis

[000248] All implants were fixed in 4% paraformaldehyde solution overnight at room temperature and then washed in PBS. The scans were performed in a micro computed tomography (micro-CT) scanner (μCT40, SCANCO Medical AG, Brüttisellen, Switzerland) at a resolution of 12 μm and three-dimensional (3D) images of implants were reconstructed from the scans by the micro-CT system software package. To validate the in vivo mineralization within the PCL scaffold, the dentin block was excluded from the scan and the average density of mineralization against hydroxyapatite (HA) was measured by the micro-CT system software package for statistical analysis between different groups.

[000249] Histology and Immunohistochemistry

[000250] Samples were decalcified in 10% EDTA at pH 7.4 for 3 months at 4 °C with a weekly change of solution and subsequently embedded in paraffin. Sections near the central area of the implants were used for the haematoxylin and eosin (H&E), Azan and immunohistochemical staining. For the immunohistochemical staining, following de-waxing and hydration, endogenous peroxidase activity within the sections was quenched by incubating with 3% H2O2 for 20 min, then blocked with 10% swine serum for 1 hour. The samples were incubated with cementum protein 1 (CEMP1) (1:200, anti-human, Santa Cruz Biotechnology Inc., USA) or alkaline phosphatase (ALP) primary antibody (1:500, anti-human, Sigma-Aldrich, Australia) overnight at 4°C, followed by incubation at room temperature with a biotinylated universal swine-anti-mouse, rabbit, goat secondary antibody (DAKO, CA, USA) for 15 min, and then with horseradish perioxidase-conjugated avidin-biotin complex (DAKO, CA, USA) for another 15 min. The antibody complexes were visualized by the addition of a buffered diaminobenzidine (DAB) substrate for 4 min. In negative control staining, the primary antibody was replaced with PBS. Mayer's haematoxylin (HD Scientific Pty Ltd., Australia) was used for counter staining.

[000251] Quantification of cementum deposition

[000252] The percentage success of cementum formation was defined as the number of samples with cell sheets which demonstrated any deposition of cementum-like tissue, divided by the total

number of samples with cell sheets. Similar calculation was performed for the groups without cell sheet. When a cementum-like tissue was observed, its percentage coverage was also calculated as the length of dentin covered with cementum divided by the total dentin length.

[000253] Statistical analysis

[000254] The statistical analysis was performed using one-way ANOVA followed by a Tukey HSD post-hoc test in case of equal variance; otherwise a Games-Howell post-hoc test was utilized. $p < 0.05$ was considered as statistically significant.

[000255] **Results**

[000256] Scaffold morphology

[000257] The fabrication technique and the morphology of the biphasic tissue graft are shown in Figure 1. During fabrication, the FDM component was held at a 1 cm distance from a hot plate which resulted in the partial melting of the bottom layer (Figure 1a). Upon press-fitting onto the electrospun component, the molten PCL transferred some heat to the underlying electrospun fibers which in turn partially melted. Upon cooling and solidification, the two components were firmly adhered and it was not possible to peel off the electrospun fibers without destroying the membrane. It was also found that the partial melting of the FDM bottom layer did not induce a significant change in the physical integrity and morphology of this component (Figure 1b). Confirmation of partial melting of the electrospun fibers is shown by the SEM cross-sectional view Figure 1c. It can be clearly seen that the upper layer of the electrospun scaffold experienced some melting which resulted in a strong connection with the FDM component as shown by the black arrow in Figure 1c. The fusion of the electrospun fiber was only localized at the interface of the FDM filaments and the other adjacent electrospun fibers remained intact which created an interconnected porous architecture between the two components.Similarly, this technique can be applied for the development of biphasic scaffold with better resilience and dimensional adaptability. This can be achieved by partially melting a melt electrospun scaffold placed at 3 cm form a hot plate for 4s and press-fitting it onto a solution electosprun membrane.

[000258] Figure 1: Fabrication of the biphasic tissue graft. a) fabrication scheme, b) and c) cross-sectional views of the biphasic tissue graft by scanning electron microscopy showing the fusion of the electrospun fibers onto the FDM component.

[000259] In vitro study

[000260] The first step of the in vitro study was to characterise the capacity of both cell types (osteoblasts and periodontal ligament cells) to deposit mineralized matrix on a 2D surface. For this purpose the cells were cultured in basal and osteogenic media for up to 14 days.

[000261] 2D Culture

[000262] The periodontal ligament cells (PDL) proliferated on the tissue culture surface as shown in Figure 2 a) and c). As a result of the osteoinduction a significant increase in the number of periodontal cells was observed for both time points. Osteoinduction resulted in higher levels of mineralization as seen in Figure 2 b) and for both time points more mineralized matrix was secreted for this group. Although a higher level of ALP activity was detected for the osteogenic induced cells, when this was normalized with the DNA content the trend was inverted and the basal condition showed a higher activity at day 14.

[000263] Figure 2: In vitro 2D culture of the periodontal ligament cells (a-d) and osteoblasts (e-h). a) and e) optical microscopy, b) and f) alizarin red S staining and semi-quantification, c) and g) cell proliferation as measured by DNA content assay, d) and h) alkaline phosphatase activity measured from the media Bars and stars show significant differences as measured by one way ANOVA ($p < 0.05$)

[000264] Similar experiments were performed for the osteoblasts used in this study and the same trend was observed for both DNA content and alizarin red S staining throughout the 2D culture. Indeed, the osteoinduction resulted in a significant increase in the cell number (Figure 2 e) and g)) a higher level of mineralization (Figure 2 f)) and in this case, the ALP activity was significantly higher at day 14 compared with basal conditions (Figure 2 h)).

[000265] These in vitro studies assessed the performances of the two primary cell lines and showed they were suitable for the purpose of engineering both the periodontal ligament and the alveolar bone.

[000266] 3D culture - Osteoblasts

[000267] Osteoblasts were cultured in the biphasic tissue graft for up to 21 days under basal or osteogenic conditions. As shown in Figure 3, the FDM scaffold, called herein bone compartment, supported cell ingrowths as the DNA content gradually increased between each time point. The basal condition did not display such a clear trend as no significant increase was observed for all time points.

[000268] Figure 3: DNA content of osteoblasts within the biphasic tissue graft. Bars and stars show significant differences as measured by one way ANOVA ($p < 0.05$).

[000269] As shown in Figure 4, unstimulated osteoblasts mostly grew around the polymer struts and no significant difference in the morphology of the seeded tissue grafts could be observed between the different time points (Figure 4 a)-f)). Confirmation of the induced osteoblasts proliferation was obtained by SEM (see Figure 4). The induced osteoblasts were capable of spanning the nearby struts (Figure 4g) and j)) and thus they created bridges between the different layers of the bone compartment. As a result, the osteoblasts gradually colonized the pores of the bone compartment (Figure 4 g)-i)) until the pores were completely filled by day 21 (Figure 4 l)).

[000270] Figure 4: SEM of the osteoblast-seeded biphasic tissue graft. The osteo-induced otsteoblasts gradually filled the pores of the bone compartment.

[000271] Confocal laser microscopy revealed similar findings. Figure 5 shows that induced cells bridged the surrounding polymer struts (Figure 5 g)-i)) whereas for the basal media the osteoblasts covered the struts without creating any connections with the other struts. Interestingly, the osteoblasts in both cases (basal or osteogenic media) could migrate to the other side of the periodontal compartment as shown in Figure 5 d)-f) and j)-l). Throughout the culture period the osteoblasts did not form a dense cell sheet when cultured in basal media but the osteo-induced cells were capable of fully colonizing the surface of the periodontal compartment and created a cell sheet only after 14 days of culture. Confirmation of this was found upon SEM-imaging the periodontal compartment (data not shown).

[000272] Figure 5: Confocal imaging of the osteoblast seeded biphasic tissue grafts. The nuclei are stained blue and the actin filaments red.

[000273] The 3D in vitro culture therefore showed that the biphasic tissue graft promoted the growth of osteoblasts in the bone compartment and that this compartment was filled with cells after 21 days.

[000274] 3D culture – Periodontal ligament cells

[000275] The rationale for using an electrospun membrane for the periodontal compartment originates from the design requirement of a strong cell sheet attachment onto this compartment before implantation in order to reduce the risk of biomechanical instability resulting in cell sheet displacement and subsequent failure to achieve cementogenesis and new attachment formation. The cell sheets were folded over the edges of the periodontal compartment to enhance the stability of the sheet and to provide additional anchorage points to ensure that the sheet did not contract or move (Figure 6 a) and b)). Finally, the flexibility of the periodontal compartment allowed the cell sheets to accurately and intimately adapt to the root surface when implanted.

[000276] In this study, the cell sheet was formed after 7 days of culture under osteogenic conditions and could be harvested because of their partial spontaneous contraction. Therefore, no thermo-responsive plates were utilized in the present study. As shown the Supplementary figure 1a), after a certain culture time, the cells secreted significant amount of protein and exerted forces onto the extracellular matrix which resulted in local contraction starting from the edge of the well. The inventors have experimentally observed that 7 days were necessary for initial contraction of the PDL ovine cells to occur. At this stage, the risk of damaging the sheet upon handling was low if not negligible. Figure 6 (c) shows that cell viability was not affected by this method of cell sheet harvesting as most of the cells were stained green (indicating that they were viable) and only a few were stained in red (dead). Under SEM imaging the cells displayed a healthy morphology (smooth membrane), they were densely packed (Figure 6 d)) and had secreted a significant amount of mineralized matrix (Figure 6 b), d)-e)). A cross-sectional view of the PDL compartment shows that the cell sheet formed a thick multilayered cellular tissue in which the different layers appear to be integrated (Figure 6 e)).

[000277] Figure 6: Multiple cell sheets onto the electrospun membrane (periodontal component) of the biphasic tissue graft. a) and b) anchorage points on the periodontal compartment, c) live dead assay, green staining indicates living cells as opposed to red which indicates dead cells , d) morphology of the last cell sheet, e) thickness of the three cell sheets.

[000278] In vivo study

[000279] In order to demonstrate the simultaneous regeneration of alveolar bone and periodontal ligament, as well as cementogenesis and periodontal attachment formation, the biphasic tissue graft/cell constructs were subcutaneously implanted into an athymic rat model. Before implantation the TECs were placed onto dentin block and fixed using sutures as shown in Figure 7. The postoperative course of the five animals was mostly uneventful during the healing period except that two samples were slightly exposed 7 days post-implantation. However this did not compromise the results since only a small fraction of the dentin block (not the tissue grafts) was exposed. Eight weeks post-implantation, the animals were sacrificed and all constructs collected. The tissue grafts exhibited good tissue integration following implantation in the subcutaneous pockets (Figure 7) and no adverse events such as foreign body reaction or infection were observed. Throughout the duration of the implantation no detachment of the biphasic tissue graft from the dentin block was observed for all groups demonstrating the high stability of this construct.

[000280] Figure 7: Description of biphasic tissue graft assembling onto the dentin block and illustration of the subcutaneous implantation in athymic rats.

[000281] Mineralization within the scaffolds was analyzed by micro-CT. However, it was not possible to directly compare the bone volume between the different samples due to the intrinsic and unpredictable differences in the bone compartment dimensions and hence sample volume. Therefore the bone volume was normalised by the total volume of the sample which enabled a meaningful comparison. Figure 8 shows the 3D reconstruction of the biphasic tissue graft onto the dentin block. Some small mineralized nodules could be observed in both the control and osteoblast induced groups. Upon quantifying the mineralization, a significant difference in the bone density was observed between empty and osteoblast seeded tissue grafts as shown in Figure 8. However, no clear differences were found between the groups with or without cell sheets and with or without osteo-induction.

[000282] Figure 8: Micro-CT of the biphasic tissue grafts 8 weeks post-implantation in a subcutaneous athymic rat model. Bars show the statistical difference between control (empty group) and osteoblast seeded scaffold (OB and OB induced)

[000283] To demonstrate the osteogenic potential of the bone compartment, ALP immunohistochemical staining was performed. This showed that endogenous ALP from cellular infiltrate was located mostly around the polymer struts of the bone compartment for the empty group (Figure 9 b)), indicating a certain level of osteogenic induction from the polymer scaffold. Endogenous ALP was also expressed by the surrounding connective tissues but the staining was less intense. However, the ALP staining was significantly more intense in the osteoblast seeded scaffolds around the polymer struts (Figure 9 c) and d)) with compared with the empty scaffold. The intensely stained tissue was thicker in most locations in the case of osteoblast seeded tissue grafts, irrespective of the culture conditions (basal or osteoninductive). These findings were agreement with the micro-CT data that demonstrated higher bone density in the scaffolds seeded with osteoblasts.

[000284] Figure 9: Histological findings a)-d) Alkaline Phosphatase staining of the bone compartment. a) negative control staining, b) empty scaffold, c) osteoblasts seeded scaffold, d) osteoblasts induced scaffold, e)-r) periondontal compartment, e)-h) and i)-l) representative H&E and Azan staining at the interface with the dentin block in the presence or absence of multiple cell sheets, m)-r) CEMP1 staining at the interface with the dentin in the presence and absence of cell sheets. Black arrows show the location of the positive CEMP1 staining.

[000285] In this study, ectopic periodontal regeneration was mediated by the implantation of multiple PDL cell sheets at the interface with a dentin block. Although good in vivo cell colonization was observed throughout the electrospun membrane (periodontal compartment) regardless of the presence of the PDL cell sheets, no tissue attachment onto the dentin was observed in the groups without cell sheets (Figure 9 e) and f)). In contrast, periodontal fiber attachment was obtained for the groups with cell sheets as shown in Figure g) and h) with cementum-like tissue of various thickness noted on the dentin surface. Indeed, most cell sheet containing samples (67%) exhibited cementum like tissue on the dentin surface whereas the group without cell sheets displayed cementum formation in only 17% of samples (Table 1). Table 1 also depicts the extent of cementum root coverage (%) which was always significantly higher for the tissue grafts with PDL cell sheets. Interestingly, small blood vessels were also found in the periodontal compartment for all groups indicating that the electrospun material was not an impermeable tissue barrier and that the cell sheets, when implanted, could be supplied as in their natural niche by a microvascular network during the healing process.

Table 1: Quantification of cementum deposition

	Cell sheets	No cell sheet
success rate %	67	17
Dentin overage %	65±22	33±4

[000286] Similarly, the azan staining showed the presence of a mineralized tissue resembling cementum associated with the tissue grafts containing the PDL cell sheets. A good example of this tissue is shown in Figure 9 k) and l) which is in contrast to the control tissue grafts without cell sheets where no cementum like tissue deposition was observed on the root surface (Figure 9 i) and j)). This cementum-like tissue covered almost entirely the dentin width.

[000287] CEMP1 staining revealed the presence of this cementum specific protein in the mineralized deposition on the surface of the dentin as shown in Figure 9 m)-r). CEMP1 was also observed in the PDL compartment as depicted in Figure 9 o) and q), which suggests the presence of cementoblast precursors.

Example 2. Calcium Phosphate loading of biphasic tissue grafts

[000288] In this example a biphasic tissue graft was built by attaching a fused deposition modeled bone compartment to a melt electrospun scaffold as described in example 1 above. The effects of coating a scaffold corresponding to a bone compartment were assessed.

[000289] **Materials and Methods**

[000290] Biphasic tissue graft fabrication

[000291] Bone compartment

[000292] Medical grade polycaprolactone (PCL) containing β-tricalcium Phosphate (β-TCP, 20% wt) was utilized to fabricate composite tissue grafts via Fused Deposition Modeling (FDM, Osteopore Inc., Singapore). The scaffolds measured 100x100x2 mm^3 and had 100% interconnectivity, 70% porosity and a 0/90 degrees lay-down pattern. Prior to use the FDM scaffold block were sectioned with a sharp scalpel blade into 5x5x2 mm^3 specimens.

[000293] The FDM scaffolds were submitted to a calcium phosphate coating process by successive immersion into specific reagents and solutions. The procedure consisted of the following steps: immersion in 100% ethanol for 15 minutes under vacuum, immersion in sodium hydroxide 2 M for 30 min at 37ºC, multiple rinse-immersions in ultrapure water until a water pH of 7 is reached, immersion in a 10x simulated body fluid (SBF) solution (solution preparation described elsewhere (Yang, F., Wolke, J. G. C. & Jansen, J. A. (2008) Biomimetic calcium phosphate coating on electrospun poly(ε-caprolactone) scaffolds for bone tissue engineering. *Chemical Engineering Journal* **137,** 154–161) for 30 min at 37ºC and immersion in a 0.5 M sodium hydroxide solution for 30 min at 37ºC. Finally the coated scaffolds were rinsed with ultrapure water and stored in a desiccator until use.

[000294] Periodontal compartment

[000295] Medical grade polycaprolactone (PCL, Lactel, USA) was electrospun using an in-house melt electrospinning device. Polymer pellets were loaded into a 2 mL syringe and electrospun at a temperature of 80°C at a feed rate of 20 µL/h, at 7 kV and at a 4 cm tip to collector distance. Circular membranes with 8 mm diameter were produced by electrospinning the molten PCL for periods of 4 min onto aluminum foil-covered glass slides placed over the collector.

[000296] Assembly of the biphasic tissue graft

[000297] The assembly of the biphasic tissue grafts was performed accordingly to the protocol outlined in Example 1. Briefly the FDM component was placed 1 cm from a hot plate heated to 300°C for 4 seconds and then quickly press-fitted for 10 seconds onto the PCL melt-electrospun membrane. This heat treatment partially melted the first layer of the FDM component enabling it to strongly bind to the electrospun scaffold upon cooling and solidification.

[000298] Biphasic tissue graft characterization

[000299] Scanning electron microscopy (SEM)

[000300] SEM was used to assess the scaffold morphology as well as to evaluate the cohesion of the two compartments. The tissue grafts were immersed in liquid nitrogen for 5-10 min and a

sharp razor blade was used to section the structures. The samples were gold coated for 3 min and observed with a FEI Quanta 200 Environmental SEM operating at 10 kV.

[000301] X-ray diffraction

[000302] The CaP coating was characterized by X-ray diffraction using a PANalytical X'Pert MPD Powder X-ray Diffractometer, a Cobalt anode and a 2 theta step size of 0.001.

[000303] In vitro study

[000304] Cell isolation and culture

[000305] Osteoblast and periodontal ligament explants were obtained from Merino sheep (ovis aries). Animal ethics approval for this study was granted by the Animal Ethics Committee of the Queensland University of Technology.

[000306] Osteoblasts

[000307] Compact bone samples were collected under sterile conditions from the mandible under general anesthesia with a trephine drill (5 mm diameter), minced, washed with phosphate buffered saline (PBS) and vortexed 5 times. Bone samples were then incubated with 10 mL of 0.25% trypsin/EDTA for 3 min at 37°C in a 5% CO_2 atmosphere. After trypsin inactivation, samples were washed once again with PBS and transferred in basal culture media (DMEM containing 10% FBS, 1% of penicillin/streptomycin) into 175 cm_2 tissue culture flasks. Outgrowth of osteoblasts was observed after 5-7 days. Cells were expanded and used at the third passage (P3).

[000308] Periodontal ligament cells

[000309] Two incisors were extracted and placed into a 50 mL tube containing DMEM with 2% penicillin/streptomycin and 4 µg/mL fungizone. The middle third of the periodontal ligament (PDL) was subsequently gently removed from the root surface with a scalpel and further sectioned into approximately 1x1 mm^2 pieces. The PDL tissues were placed into a 25 cm^2 culture flask which was left standing upright in an incubator at 37°C and 5% CO_2 atmosphere for 30 min to allow tissue adhesion. After this incubation period, 3 mL of DMEM containing

10% FBS, 1% of penicillin/streptomycin and 0.1 μg/mL fungizone were added and the flask was carefully laid down in the incubator. The first media change occurred 4 days post extraction. After one week of culture, cells started migrating outwards from the PDL tissues and reached confluence after 2-3 weeks of culture. The cells were passaged using 0.25% trypsin and further expanded until P3.

[000310] Biphasic tissue graft seeding and culture

[000311] Osteoblasts (200,000 cells in 40 μL of media) were seeded onto the FDM component and allowed to adhere for 4 hours at 37°C in a 5% CO_2 atmosphere before the well was filled with media. The biphasic tissue grafts were then turned upside down with the periodontal compartment facing upwards in order to minimize cell infiltration into the electrospun component. Biphasic tissue grafts were further cultured for 6 weeks either in osteogenic media (50 μg/mL ascorbate-2-phosphate, 10 mM β-glycerophosphate, 0.1 μM dexamethasone) or in basal media. The biphasic tissue grafts were entirely covered by the medium. Four groups were created: Non-coated with osteoblasts in basal media (N-N), or in osteogenic media (N-O), CaP coated scaffold with osteoblasts in basal media (CaP-N), or in osteogenic media (CaP-O). Osteoblast proliferation and alkaline phosphatase activity were measured at 2, 4 and 6 weeks post seeding according to the procedure described in the following section.

[000312] Alkaline phosphatase activity and DNA content

[000313] ALP activity was measured from the media in triplicate at different time-points (2, 4 and 6 weeks) after a 24 hours release period as described in the supplementary information. DNA content was measured utilizing the Picogreen kit after matrix digestion in a solution of proteinase K (more details can be found in the Supplementary Information). For the ALP and DNA analyses, 4 biological replicates per group were used.

[000314] Scanning electron microscopy (SEM)

[000315] Osteoblasts morphology and distribution into the biphasic tissue graft were assessed at 2, 4 and 6 weeks post-seeding. Samples were fixed in 3% glutaraldehyde until processing. Samples were treated by sequentially immersing them into 0.1 M cacodylate buffer, 1 % osmium tetroxide in cacodylate buffer, rinsed in ultrapure water and then dehydrated by immersion into increasingly higher concentration ethanol solutions. Finally, samples were

immersed in hexamethyldisilazane for 60 min, air dried, mounted onto stubs and gold coated. Samples were observed on a FEI Quanta 200 Environmental SEM operating at 10 kV.

[000316] Confocal laser microscopy

[000317] Cellular morphology and orientation were assessed by confocal laser microscopy at 2, 4 and 6 weeks post-seeding. Samples were washed in PBS and fixed overnight in 4% (w/v) paraformaldehyde (PFA)/PBS. Samples were then washed in PBS and permeabilized with 0.2 % Triton X100/PBS for precisely 5 minutes and again washed twice with PBS. F-actin filaments and nuclei were then stained for 45 minutes with 0.8 U/mL rhodamine 415-conjugated phalloidin and 5 μg/mL 4'-6-diamidino-2-phenylindole (DAPI) respectively in PBS. Immunofluorescence was visualized and z-stacks acquired using a confocal microscope (SP5, Leica).

[000318] Micro-CT analysis

[000319] The effect of the CaP coating along with the *in vitro* culture conditions upon mineralization was quantitatively assessed 6 weeks post seeding by micro-computed tomography. Prior to cell seeding each biphasic tissue graft was assigned a number to allow sample tracking. They were thereafter scanned in a micro-computed tomography (micro-CT) scanner (μCT40, SCANCO Medical AG, Brüttisellen, Switzerland) at a resolution of 12 μm, a voltage of 45kVp and a current of 177 mA in order to obtain the signal originating from the scaffold only (called herein scan 1). At the end of the *in vitro* culture (6 weeks post-seeding) these samples were fixed in 4% paraformaldehyde solution overnight at room temperature and then washed in PBS and scanned again according to the same parameters in order to measure the mineralization volume (referred to as scan 2). The true mineralization was quantitatively determined by subtracting the corresponding initial signal (scan 1) from the mineralization volume at the end of the culture (scan 2).

[000320] In vivo study

[000321] The ability of the biphasic tissue graft to facilitate periodontal regeneration including simultaneous bone, periodontal ligament and cementum regeneration was assessed following insertion in ectopic (subcutaneous) sites in rats. The osteoblast loaded tissue grafts were cultured for 6 weeks and then three PDL ligament cell sheets were successively placed onto the melt

electrospun PCL membrane, thus creating the periodontal compartment. The following sections describe the cell sheet harvesting along with the animal implantation.

[000322] Harvesting of cell sheets

[000323] PDL cells were seeded at 10,000 cells/cm2 in a 24-well plate. The cells were cultured in osteogenic media and as the cell sheet matured (after 7 days of culture), it started to contract and detach from the well. Three PDL cell sheets were then harvested using the electrospun component of the biphasic tissue graft.

[000324] Dentin slices and tissue graft assembly

[000325] One millimeter thick dentin slices were prepared from sheep teeth and adjusted to the size of the biphasic tissue grafts. The assembly of the biphasic tissue grafts onto dentin blocks was performed under sterile conditions and sutures were used to keep the biphasic tissue graft on top of the dentin slice. Thereafter the samples were immersed in media for 2 hours in order to allow for cell sheet adhesion onto the dentin surface. Notches on the dentin slices were created in order to prevent the sutures from sliding off, thus ensuring high stability of the tissue graft.

[000326] Subcutaneous implantation

[000327] Animal ethics approval for the use of athymic nude rats in this experiment was granted by the Animal Ethics Committee of Griffith University. Five 8-week old male rats (Animal Resources Centre, Canning Vale, WA, Australia) were used. The animals were anaesthetized with isoflurane. Six small incisions were made longitudinally along the central line of the shaved dorsal area, approximately 2 cm apart, and subcutaneous pockets were made with surgical scissors. Six different groups of biphasic tissue grafts were implanted for 8 weeks. The groups consisted of 1) non coated biphasic tissue grafts combined with cell sheets (n=5) or 2) without cell sheets (n=5); 3) non-coated biphasic tissue grafts with osteoblasts cultured in basal media (n=5) or 4) in osteogenic media (n=5); 5) CaP-coated tissue grafts with osteoblasts cultured in basal media (n=5) or 6) in osteogenic media (n=5). Each individual pocket held one tissue graft and the incisions were closed with 5/0 silk sutures. Therefore, 4 groups out of 6 did not receive the CaP coating. The animals were sacrificed after eight weeks and the implants were retrieved and fixed in 4% paraformaldehyde in PBS at pH 7.4 for further analysis.

[000328] Micro-CT analysis

[000329] To determine the efficacy of the biphasic tissue graft in regard to mineralized tissue formation, the retrieved samples were analyzed by micro-CT according to the parameters described in section 2.3. Here again the intrinsic signal originating from the tissue graft was measured prior to in vitro culture and subtracted from the signal obtained when scanning the samples 8 weeks post implantation. This permitted the precise measurement of the amount of newly formed bone. To validate the in vivo mineralization within the PCL scaffold, the attached dentin block was excluded from the scan and the average density of mineralization against a hydroxyapatite standard was measured by the micro-CT system software.

[000330] Histology

[000331] Samples were decalcified in 10% EDTA at pH 7.4 for 3 months at 4 °C with a weekly change of solution and subsequently embedded in paraffin. Sections near the central area of the implants were used for the haematoxylin and eosin (H&E) staining. Periodontal-like attachment was defined as direct contact of the connective tissue (or cell sheets) with the dentine slice. The number of specimens demonstrating periodontal-like attachment in each group (with or without cell sheets) was calculated and compared to the total number of samples within the same group.

[000332] Statistical analysis

[000333] The statistical analysis was performed using a Shapiro-Wilk normality test and one-way ANOVA followed by a Tukey HSD post-hoc test. $p < 0.05$ was considered as statistically significant.

[000334] **Results**

[000335] Tissue graft morphology

[000336] Figure 10 depicts the global strategy developed in this study from the tissue graft fabrication and assembly to the *in vivo* implantation. The bone compartment was coated with a layer of calcium phosphate homogenously distributed on the polymer struts. This surface modification displayed the typical features of biomimetic calcium phosphate coating, that is formation of cauliflower-like structures as seen in Figure 11(B). The NaOH pre-treatment

resulted in exposure of the β-TCP particles on the surface of the PCL struts. This enabled the creation of specific nucleation sites for the deposition of the CaP coating. Indeed, the calcium phosphate preferentially nucleated onto the TCP particles (probably due the higher affinity and the lower surface energy) and then spread over the surface on the PCL strut.

[000337] The resulting CaP coating was composed of a mixture of Di-Calcium Phosphate Dihydrate (DCPD) and carbonate apatite, however the NaOH post treatment was capable of removing the more soluble phase (DCPD). Indeed, the XRD analysis (Figure 11 (E)) revealed that the intensity of the peaks corresponding to DCPD was reduced after the post-treatment while the intensity of the peaks corresponding to carbonate hydroxylapatite was increased. This indicates that the CaP deposits on the polymeric strut had good physical stability, which is necessary for osteogenesis. Upon sectioning of the bone compartment, it was possible to access the approximate thickness of the coating. The SBF immersion resulted in the deposition of a 600-800 nm coating.

[000338] The periodontal compartment consisted of a melt electrospun scaffold composed of randomly orientated PCL fibers. As seen in Figure 11(D), fiber fusion occurred at different locations, which created a concentric ring pattern during the fabrication process. The fiber diameter was around 10 to 15 μm which resulted in the formation of a fully interconnected porous structure, with pore sizes ranging from 100 to 400 μm (as estimated by SEM). The assembly of the biphasic tissue graft was performed through a press-fit methodology by compressing the partially fused CaP coated bone compartment (FDM scaffold) onto the periodontal compartment (melt electrospun mesh) for several seconds. Upon solidification of the partially molten bone compartment, both scaffold remained fused and firmly attached together. As seen in Figure 11 the tissue graft assembly was not detrimental to the physical integrity of each individual component.

[000339] In vitro study

[000340] Cell imaging

[000341] Osteoblasts were seeded and cultured *in vitro* in the biphasic tissue graft's bone compartment for a total period of 6 weeks. Confocal laser microscopy and SEM imaging (Figure 12) revealed that 2 weeks post-seeding cells were homogenously distributed around the bone compartment's polymer struts and acquired a spindle-like shape. Noticeable differences were

observed between osteoblasts grown in the groups cultured in basal media (N-N and CaP-N) and the ones under osteogenic induction (N-O and CaP-O) as the latter were capable of spanning between the polymer struts and therefore started filling the macroscopic pores of the bone compartment. Notably, the periodontal compartment was also infiltrated by the osteoblasts as early as 2 weeks post seeding regardless of the culture conditions. The cells entirely filled the melt electrospun pores after 2 weeks of osteogenic induction and formed a dense cell layer on the surface of the periodontal compartment. A similar effect was observed for the cell in basal media albeit at a later time point (6 weeks post seeding). Scanning Electron Microscopy also revealed that the osteoblasts accumulated at the interface of both compartments when cultured under osteogenic induction (second and fourth column).

[000342] DNA content and Alkaline Phosphatase activity

[000343] Cell proliferation was assessed by DNA quantification. At two weeks post-seeding, a significantly higher amount of DNA was found in the groups under osteogenic induction but this effect was less pronounced for the two later time points (Figure 13(A)). It was also observed that the osteoblasts in basal media, seeded onto the non-coated scaffold (N-N group) supported significant cell proliferation at 6 weeks post-seeding, whereas the DNA remained constant for the other experimental groups excepted for the N-O group in which a gradual but significant decrease was observed (Figure 13(B)). The reason for this finding is unclear but it may be linked to a lack of diffusion of oxygen and nutrient in the depth of the tissue graft due to the formation of a thick cellularized tissue at the periodontal/bone compartment's interface which may have caused, to some extent, unexpected cell death.

[000344] An ALP quantification assay was used to measure the osteogenic activity of the osteoblasts seeded into the biphasic tissue graft at 2 week intervals over 6 weeks of *in vitro* culture. At 2 weeks post seeding, the CaP coating resulted in significantly enhanced ALP activity in the group CaP-N (cultured in basal media, Figure 13(C)). A noticeable increase in ALP expression was observed in the osteo-induced samples (N-O and CaP-O) although it did not reach statistical significance. Notably, the CaP-N group displayed higher ALP expression at 2 weeks post seeding than the osteoblasts cultured in osteogenic media, exemplifying the efficacy of the CaP coating at enhancing ALP activity. The synergetic effect of the coating and the osteogenic induction was more pronounced at 4 weeks post seeding where the CaP-O group induced significantly higher ALP expression than any other groups. ALP activity was

significantly up-regulated at 6 weeks post-seeding in both groups with osteogenic media when compared the samples in basal media (N-N and CaPN). However no statistical difference was found between N-O and CaP-O at this time point. Figure 13(D) depicts ALP activity for each group throughout the time course of the *in vitro* culture, and it can be observed that ALP activity remained constant in the samples cultured in basal media whereas the osteogenic induction resulted in a gradual and significant increase in this parameter.

[000345] Micro-computed tomography

[000346] The volume of mineralized matrix deposited by the cells during the 6 weeks of *in vitro* culture was quantified by micro-CT and is shown in Figure 13(E). It was observed that the CaP coating clearly resulted in enhanced mineralization within the tissue grafts regardless of the culture conditions. More importantly, it showed that osteogenic induction was the most potent signal for secreting mineralized matrix as both, N-O and CaP-O groups displayed much higher mineralization volume than the corresponding groups cultured in basal media. Indeed, mineralization in N-N samples remained within the noise level, whereas 1.9 and 2.5 mm^3 of mineralized matrix was produced for N-O and CaP-O respectively. This corroborated the ALP activity profile observed in these groups and confirmed the beneficial effect of CaP coating on the deposition of mineralized matrix. The average mineralization density, as assessed from the micro-CT scan, provided insights into the mineralized matrix maturation, whereby higher density is associated with a more mature matrix. However no significant differences were observed between CaPO and NO suggesting that the mineralized matrix was equivalent "quality".

[000347] In vivo study

[000348] After an uneventful 8 weeks post-operative period, the animals were sacrificed and the constructs collected. Good tissue integration into the subcutaneous pocket was observed and no acute foreign body reaction or infections were observed.

[000349] The amount of newly formed bone in the constructs was analyzed using micro CT (Figure 14(A)). The results showed that the samples cultured in basal media did not form any bone, with the signal remaining at the background level. In contrast, the samples cultured in osteogenic media resulted in bone formation and CaP-O displayed the highest level of bone (Figure 14(A)). Figure 14(B) displays the density of the newly formed bone and it was observed

that CaP-O specimens featured the highest density when compared to all other groups. This indicates that the bone formed in the CaP coated tissue graft was denser and therefore more mature. By performing 3D reconstructions (Figure 14(C)) of the analyzed constructs, it was observed that a significant amount of new bone was located at the periodontal/bone compartment's interface. This was confirmed by the histology as shown in Figure 15. Bone formation solely occurred in samples previously cultured in an osteoinductive environment (Figure 15 (B)).

[000350] Histological analysis of the periodontal compartment revealed that the host tissue had entirely colonized the melt electrospun scaffold. It was also observed that the constructs with cell sheets (Figure 15(B)) had attached more frequently onto the dentin block compared to the constructs without cell sheets (Figure 6.a.). Despite signs of connective tissue adhesion on the dentin in the constructs without cell sheets, this attachment was not functional and was not sufficiently strong to withstand histological processing and sectioning hence generating a space between these two elements. The percentage of attachment for each condition was calculated and showed that most of the constructs with cell sheets (4/5-80%) were able to attach as opposed to 1/5-20% for the constructs without cell sheets. Blood vessels penetrated not only throughout the bone compartment (FDM scaffold) (Supplementary Figure 3.c. and e.), but also throughout the periodontal compartment (Figure 15(D)). Blood vessels were found in close vicinity of the implanted cell sheets Figure 15(D)), which may have a significant impact on cell sheet survival and subsequent tissue remodeling. It is also noteworthy that, due to the intrinsic architecture of the melt electrospun scaffold, which consisted of superimposed concentrically oriented rings, some level of tissue organization was observed as shown in Figure 15 (C). This tissue was obliquely orientated in regard to the dentin block and formed an angle of approximately 60 degrees.

Example 3. Decelluarization of Tissue Grafts

[000351] This study describes the fabrication of decellularized tissue grafts which retain an intact extracellular matrix and resident growth factors which facilitate cellular repopulation.

[000352] **Materials and methods**

[000353] Primary human periodontal ligament cells isolation and culture

[000354] Human periodontal ligament cells (hPDLC) were obtained using an established protocol (Ivanovski et al., (2001) *J Dent Res* 80: 1665-1671). Briefly, after obtaining institutional ethics approval (Griffith University Human Ethics Committee) and informed patient consent, extracted third molars were obtained from patients aged 17-30 years old. Diced periodontal tissues were obtained from the middle third of the roots, and explanted into 25 cm^2 flasks. The cells were subsequently grown and propagated in 175 cm^2 flasks with Dulbecco's Modification of Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), Penicillin (50units/ml) and Streptomycin (50µg/ml). Cells between the third and fifth passage were utilized.

[000355] Decellularization, immunostaining and confocal imagining of cultured cell monolayers

[000356] In order to characterize the structure of the cell monolayer extracellular matrix, the cells were seeded at a density of 2 x 10^4 cells on 13 mm diameter Thermanox coverslips (Thermo Scientific NuncTM, Australia) and grown for 9 days in culture medium supplemented with ascorbic acid (100 µg/ml). At the end of culture period, a cell monolayer was formed and decellularized according to the following protocol. Briefly, 800 µl of NH4OH (20mM) and Triton X-100 (0.5%) were added to the cover slips for 20 minutes and kept at 37°C. The coverslips were then rinsed twice with 1 ml of sterile water and kept in phosphate buffer saline (PBS) at 4°C.

[000357] Fresh and decellularized cell monolayers were imaged using confocal laser microscopy. Antibodies against human Collagen I and Fibronectin (Life Technologies, Invitrogen) were used to visualize the extracellular matrix. 4',6-diamidino-2-phenylindole (DAPI, 5 µg/ml) and Phalloidin – tetramethylrhodamine B isothiocyanate conjugate (Phalloidin-TRITC, 0.8 U/ml, life technologies, Invitrogen) was utilized to stain the nuclei and the actin fibers. A detailed protocol is included in the supplementary information.

[000358] Melt Electrospun carrier scaffold fabrication

[000359] Polycaprolactone (PCL, CAPA®6400 Perstorp UK) was utilized for fabricating the carrier membranes utilizing a house built melt electrospinner on a static flat collector. The PCL granules were loaded into a 2 ml syringe and melt electrospun at 6 kV, 95°C, at a feed rate of 20 µl/hr with a spinneret-collector distance of 4 cm. A biopsy punch was used to produce 5mm

diameter membranes. To enhance the scaffold hydrophilicity, a 2M NaOH treatment was performed for 30 minutes followed by 5 rinses in ultrapure water. The membranes were sterilized by immersing them in ethanol for 30 min followed by a 30-min UV irradiation. The membranes were used to support the cell sheet during the handling and decellularization process.

[000360] Cell sheet harvesting

[000361] hPDL cells were seeded in 24 well cell culture wells at a seeding density $5x10^4$ cells/well in media supplemented with ascorbic acid (1000 µg/ml). For the first 48 hours, the ascorbic acid concentration was ten fold greater than the standard concentration in order to enhance early extracellular matrix formation. The cells were then grown for 19 days in media supplemented with ascorbic acid (100 µg/ml), which was changed every 48 hours.

[000362] At the end of the 21 day culture period, the borders of the cell sheet were gently detached from the base of the well and pulled towards the edges of the PCL membranes using sterile fine curved tweezers. The samples were further incubated in culture media for 24 hours.

[000363] Decellularization of cell sheets

[000364] A bi-directional perfusion system developed by our group was utilized to decellularize the cell sheets. It was composed of a pump, 30 ml plastic syringe, 3 mm diameter silicone tube and 2 x 15 ml falcon tubes stacked on each other. Rapid prototyped polylactic acid (PLA) porous constructs (lay-down pattern 0/90/180) were used as separators to divide the falcon tubes into compartments and ensure the appropriate positioning and stability of the PCL scaffolds within the falcon tubes. Three scaffolds were placed in a sandwich pattern between two PLA constructs using sterile tweezers, with a maximum of 9 scaffolds decellularized at a time. The decellularization solution, consisting of 30mL of 20nM NH4OH solution with 0.5% Triton X-100, was bi-directionally perfused though the scaffold for 60 min at a rate of 1000 ml/hr with a flow inversion every 50 seconds. This was followed by perfusion in a DNase I solution (100U/ml, Invitrogen) at 37°C in $CaCl_2$ (0.9mM) and $MgCl_2$ (0.5mM) in sterile PBS for 60 minutes. The PCL membrane-cell sheet constructs were finally perfused with sterile water at 37°C for another 60 minutes.

[000365] Confocal imaging of cell sheets

[000366] The PCL membrane-cell sheet constructs were immunostained for human collagen I and human fibronectin before and after decellularization to assess the sheet integrity using the same technique described above.

[000367] DNA content

[000368] DNA content was measured for both fresh cell and decellularized samples using a Quant – iT PicoGreen kit after matrix digestion in proteinase K. For each group, 6 biological replicates were utilized and each measurement was performed in triplicate. A detailed protocol can be found in the supplementary information.

[000369] Scanning electron microscopy (SEM) of cell sheets

[000370] SEM imaging of fresh and decellularized cell sheets was performed utilizing a FEI Quanta 200 microscope. A detailed protocol can be found in the supplementary information.

[000371] Growth factor ELISA assay

[000372] ELISA assays were used to detect basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) levels in both fresh and decellularized samples. Growth factor extraction was performed by adding 300 µl of NaCl (2M) in 20 mM HEPES with EDTA protease inhibitor cocktail (Roche complete mini, Roche Applied Science, Indianapolis, IN) to each sample and incubated for 60 minutes at room temperature with gentle shaking on an orbital shaker. Samples were collected into 1.5 ml Eppendorf tubes and centrifuged at 2000 rpm for 5 minutes. Growth factor quantification was carried out using a Bioplex assay (Bio-Plex Pro, Bio-Rad) according to the manufacturer's instructions.

[000373] Collagen quantification:

[000374] Collagen content in the cell sheets was measured in both fresh and decellularized samples, using a hydroxyproline assay kit (Chondrex, Inc. - catalog#6017) according to the manufacturer's instructions. A detailed protocol can be found in the supplementary information.

[000375] Recellularization of decellularized sheets

[000376] Decellularized sheets were assessed for their recellularization potential by seeding of allogenic hPDL cells on the top of the decellularized constructs at a seeding density of 5000 cells/scaffold. Recellularization was assessed over 3, 7 and 21 days with confocal imaging, SEM imaging and DNA quantification using PicoGreen assay, using the same methods outlined above.

[000377] Statistical analysis

[000378] Results were expressed as ± mean standard deviation and student T test was used to analyze the data. The significance level of the statistical analysis was set at $p < 0.05$.

[000379] **Results**

[000380] Scanning electron microscopy

[000381] The incorporation of ascorbic acid in the media along with in vitro cell culture resulted in the deposition of a well-developed collagenous network and hence a mature cell sheet was formed. The sheets were thick enough after 3 weeks of culture to be mechanically harvested with fine curved tweezers. This permitted the harvesting and placement of the cell sheet onto a PCL melt electrospun scaffold (Figure 16B). Attachment of the cell sheet to the PCL scaffold was rapid, provided that the scaffolds were surface treated with sodium hydroxide to increase their hydrophilicity. It was found that a 24 hour period was sufficient for the cell sheet to become firmly adherent to the scaffold and withstand the subsequent fluid perfusion decellularization process. The SEM images revealed that both the fresh and the decellularized cell sheets remained intact and well attached to the PCL scaffold (Figure 16C and 16D). Higher magnification images of the decellularized samples demonstrated the presence of a fine network of collagen fibers with a morphology and structural integrity similar to that observed in the fresh cell sheet (Figure 16D-III & 16D-VI).

[000382] Extracellular matrix characterization

[000383] Figure 17A and 17B displays representative immunostaining of hPDLC monolayers cultured on a cover slip, showing a well-developed network of fibronectin and collagen fibers. Upon decellularization, the components of the extracellular matrix formed by the monolayers

were well preserved as shown in Figure 17C and 17D with no apparent alteration in their structural integrity when compared to the fresh matrices.

[000384] Similarly, in the case of the mature cell sheets placed on the PCL membranes, the decellularization protocol resulted in preservation of the quality and integrity of the extra-cellular matrix components (Figure 16G and 16H). Negligible traces of DNA remnants (in blue) and actin filaments (in red) were detected in the decellularized sheets, indicating efficient removal of cellular contents using this decellularization protocol.

[000385] DNA quantification

[000386] DNA quantification confirmed the efficacy of the decellularization protocol in removing the cellular components, with 92% of DNA successfully eliminated from the hPDLC sheets as shown in Figure 18A.

[000387] Growth factors ELISA and collagen quantification

[000388] bFGF, VEGF and HGF were found to be retained in the decellularized sheets. As shown in Figure 18B, approximately 10% of the initial growth factor content in the fresh cell sheet remained after decellularization. Collagen quantification revealed increased collagen content in the decellularized cell sheets, indicating that this decellularization method did not affect the amount of retained collagen (Figure 18C).

[000389] Cell growth on decellularized sheets

[000390] The decellularized matrices were re-seeded with allogenic hPDL cells and cultured in vitro over 21 days. The recellularized constructs showed a gradual and significant increase in DNA content indicating that the matrices were capable of supporting cell adhesion and proliferation as shown in Figure 4c. The cells adopted a spindle-like morphology (black arrows) as seen by confocal laser microscopy (Figure 4A) and SEM (Figure 4B). Newly formed extracellular matrix was observed at the later time points (Figure 4A-III and B-VI) indicating excellent cyto-compatibility of the decellularized substrate.

Example 4.Antibiotic Loading of Tissue Grafts

This study describes the loading and release profile of antibiotic coated tissue grafts and their behaviour in vitro and in vivo. A broad range of azithromycin doses spanning from 50 μg to 5000 μg was further investigated for its loading efficiency and release profile in order to meet various therapeutic needs. These doses are divided into two groups: the high doses (1000 to 5000ug) and low doses (50 μg to 500 μg) hence can be utilized according to the severity of the infection or potential infection.

[000391] Azithromycin loading.

[000392] PCLscaffolds or PCL scaffolds submitted to a calcium phosphate coating process described in example 2 above (PCL-CaP) were loaded with different concentrations of azithromycin.

[000393] The loading of the antibiotic is performed according to the procedure involving the dissolution of the drug into ethanol, immersion of the scaffold into this solution followed by the evaporation of the solvent. The release profile of the antibiotic was investigated over 14 days for high dose and 7 days for low dose by immersing the scaffolds in PBS.

[000394] It was demonstrated that the release occurred in a sustainable manner according to linear slope for all doses as shown in Figure 22. However, the release rate and the total release was release was dose dependent. The lower doses (50 -1000μg)) displayed a significantly more rapid release of the drug than the higher doses (Figure 22 (b),(d)). Similarly, the total release was reached notably faster for the lower dose (90% total release at 7 days) whereas the level of release remained around 20-40 % for the higher doses. This indicates the release profile of the antibiotic can be easily tailored by varying the dose incorporated in the scaffold.

[000395] Figure 22: Loading efficiency and release profile of high dose ((a),(b)) and low dose ((c), (d)) azithromycin loaded on PCL or PCL-CaP electrospun membranes .

[000396] Contact Angle

[000397] The contact angle of non-coated and CaP-coated membranes (n =4) with different doses of azithromycin (1mg, 2.5mg and 5 mg) was measured using a FTA200 Contact Angle

and Surface Tension Instrument (Poly-Instruments Pty. Ltd., Australia). PCL is hydrophobic and upon CaP coating, becomes hydrophilic. Hydrophilicity of PCL membranes increases with increasing azithromycin dose (Figure 23 (a)). On the other hand, azithromycin coating decreases the hydrophilicity of PCL-CaP membranes.

[000398] Figure 23 (A) and (B): Contact angle measurement of PCL and PCL-CaP membranes loaded with different doses of azithromycin.

[000399] Surface Charge Analysis

[000400] The zeta potential and pH of PCL/PCL-CaP membranes with or without azithromycin (5mg) were measured using an electrokinetic analyzer (SurPass, Anton Paar GmbH, Austria) and the adjustable gap cell. The analyzer was controlled with VisioLab for SurPASS (version 2.0, Anton Paar GmbH) interface. In each zeta potential recording, two identical samples were attached to the adjustable gap cell to face each other with a gap of approximately 100 μm between them. The streaming current was measured between two Ag/AgCl electrodes placed at both sides of the samples. The measurements were performed using 1mM KCL solution as the electrolyte. The VisioLab-interface calculated the zeta potential from the streaming current measurements according to the HelmholtzSmoluchowski equation (1)

$$\text{Zeta Potential} = (dI/dp) \times [\eta/(\varepsilon \times \varepsilon 0)] \times (L/A) \quad (1)$$

where dI/dp is the slope of the streaming current versus pressure, η is the viscosity of the electrolyte, ε is the dielectric constant of the electrolyte, εo is the vacuum permittivity. L is the length and A is the cross-section of the streaming channel.

[000401] It was observed that PCL and PCL-CaP membranes are highly negatively charged and azithromycin coating reduces the negative charge of the membrane. Also, azithromycin coating makes the membranes slightly basic.

[000402] Figure 23 (c): Zeta Potential and pH of PCL and PCL-CaP membranes with or without azithromycin (5mg).

[000403] Chemical Analysis

[000404] X-ray diffraction (XRD) studies were conducted on a PANalytical X'Pert PRO MPD Powder X-ray Diffractometer equipped with Cu-K α source (40 kV, 40 mA) in the range of5_e50_ at a scan rate of 5_/min.

[000405] It was seen that azithromycin is more crystalline in nature and upon dissolving in ethanol become more amorphous. Hence, azithromycin loaded PCL and PCL-CaP membranes were more amorphous in nature (Figure 23 (d)).

[000406] Figure 23 (d): XRD spectrum of PCL and PCL-CaP membranes with or without azithromycin.

[000407] Fourier transform infrared spectroscopy (FT-IR) was performed on a Nicolet 5700 FT-IR with a Smart Endurance Diamond ATR accessory. The spectra were collected by co-adding 64 scans at 4 cm-1 resolution, range 4000-600 cm-1. The spectra were ATR collected and displayed as absorbance spectra.

[000408] FTIR spectrum also indicates a decrease in crystallinity upon azithromycin coating on to PCL and PCL-CaP membranes. Pure azithromycin is crystalline is nature. 3600-3500 cm^{-1}corresponds to free O-H stretching indicating crystallinity. Distribution and uniformity of H-bonding in the amorphous region is poor and hence H bond rupture takes place. Decrease in the intensity means decrease in the crystallinity (Figure 23 (e)).

[000409] Figure 23 (e): FTIR spectrum of PCL and PCL-CaP membranes with or without azithromycin.

[000410]

[000411] Drug Stability

[000412] To demonstrate the stability of azithromycin coated PCL scaffolds over time, PCL-CaP scaffolds were loaded with different doses of azithromycin(1000µg, 500µg and 50µg)andapplied to *Staphylococcus aureus*agar plate growth inhibition assays at 0, 1, 3, 5, and 7 days after azithromycin loading.

[000413] It was also demonstrated that the drug loaded onto the scaffold did not degrade over time as shown in Figure 24. This was performed using a *Staphylococcus aureus* agar plate growth inhibition assay and showed that the drug remains active over 7 days of release. Figure 24 features the growth inhibition assay carried out for the 1000, 500 and 50μg) loaded scaffold and demonstrated highly efficient inhibition throughout the experiment whereas the negative control (PCL-CaPscaffold) did not result in any antibacterial activity as expected. The 2.5 and 5 mg doses displayed a similar, also identical pattern for bacterial inhibition.

[000414] Figure 24: Growth inhibition of *Staphylococcus aureus* after 20 hour incubation with 1mg (a), 500μg (b) and 50μg (c) azithromycin containing PCL-CaP electrospun membranes after 0,1,3,5 and 7 days release from PBS at 37°C.

[000415] The maintenance of the antibacterial activity until closure and healing of the wound is essential into in order to avoid adverse events such as infection. It further ensures subsequent bone growth and hence is of paramount importance in the context of guided tissue regeneration or guided bone regeneration.

[000416] In Vitro

[000417] A live dead assay was performed to determine the cell viability at various doses of azithromycin.

[000418] Briefly, PCL-CaP membranes coated with various doses of azithromycin (10, 50, 100, 250 and 500ug) and PCL-CaP membranes without azithromycin coating were first UV sterilized. Each membrane (6mm diameter) were then placed on a 48 well plate. Human osteoblasts (1000 cells in 40 μL of culture media) were seeded onto each membrane. The cells were hydrated every 30 min by adding 40μL of media. After 4 h of incubation, 150μl of media was added to the well. The membranes were cultured for 24 hours. Cell viability was observed with confocal laser microscopy by fluorescein diacetate (FDA)/propidium iodide (PI) staining. FDA stained viable cells green and PI stained necrotic or secondary apoptotic cells red. After 24 hour of incubation the cell seeded membranes were first thoroughly rinsed twice with PBS. Then 200μl of FDA_PI solution (mixing 5 μl FDA stock, 5 μl PI stock diluted in 5 ml PBS) was added. The system was allowed to incubate at 37°C for 5 min. Then the samples were washed twice with PBS and observed by confocal microscopy.

[000419] As shown in Figure 25, cell viability decreased with increasing dose of azithromycin.

[000420] Figure 25: Live dead assay on human osteoblast cultured with various doses of azithromycin coated PCL-CaP membranes (a) control group with PCL-CaP membranes alone, PCL-CaP membranes with 10μg (b), 50μg (c), 100μg (d), 250μg (e), 500μg) of azithromycin

[000421] In Vivo

[000422] In order to assess the tissue response *in-vivo*, we performed a subcutaneous implantation of PCL-CaP scaffolds loaded with various doses azithromycin was performed Sprague Dawley rats according to the procedures outlined in Examples 1 and 2.

[000423] Briefly, six small incisions were made longitudinally along the central line of the shaved dorsal area, approximately 2 cm apart, and subcutaneous pockets were made on each side of the incision with a pair of surgical scissors. Each PCL-CaPscaffold with different dose of azithromycin (0, 100μg, 250μg, 500μg, 750μg and 1mg) were placed in one subcutaneous pocket. The pocket was sutured and the animal be allowed to recover. The animals were sacrificed after one and four weeks and the implants were retrieved and fixed in 4% paralformaldehyde in PBS at pH 7.4 for further analysis.The sample size were determined by carrying out power calculations based on assumptions informed by outcomes from our previous studies with these animal model, aiming to achieve a power of 0.9 and an $\alpha<0.5$.

[000424] Histological analysis using H&E staining was performed as outlined above in Example 2. This analysisrevealed a significant decrease in early soft tissue attachment at one week post implantation (Figure 26). Percentage of soft tissue attachment calculated after one week implantation showed a significant decrease with 750μg and 1000μg of azithromycin loaded scaffolds (Figure 26(g)) when compared to 100μg and 250μg of azithromycin loaded PCL-CaP scaffolds. However, no significant difference was observed in percentage of tissue integration one week post implantation. No significant difference on soft tissue attachment and integration was observed after four weeks, indicating minimum toxicity (Figure 27).

[000425] Figure 26: Haematoxylin and eosin staining of PCL-CaP membranes loaded with 0μg (a), 100μg (b), 250μg (c), 500μg (d), 750μg (e) and 1mg (f), percentage of soft tissue attachment and integration (g) one week post-implantation in Sprague Dawley rats (n=3),* ($p<0.05$) when compared to 100μg and 250μg.

[000426] Figure 27: Haematoxylin and eosin staining of PCL-CaP membranes loaded with 0μg (a), 100μg (b), 250μg (c), 500μg (d), 750μg (e) and 1mg (f), percentage of soft tissue integration (g) four week post-implantation in Sprague Dawley rats (n=3).

CLAIMS

1. A decellularized biphasic periodontal tissue graft comprising: a first polymeric scaffold comprising a plurality of first polymer fibers, and a second polymeric scaffold comprising a plurality of second polymer fibers; wherein

said first and second polymeric scaffolds are interconnected;

said first polymer fibers are arranged such that said first polymeric scaffold has a porous structure with an average first pore size, and said second polymer fibers are arranged such that said second polymeric scaffold has a porous structure with an average second pore size;

said first polymer fibers are coated with an osteoblast-derived extracellular matrix,

and said second polymer fibers are coated with a periodontal ligament cell-derived extracellular matrix.

2. The tissue graft according to claim 1, wherein said average first pore size is greater than said average second pore size.

3. The tissue graft according to claim 1 or 2, wherein said first scaffold comprises a fixed deposition molded polymer or a melt-electrospun polymer, and said second scaffold comprises a melt-electrospun polymer or a solution-electrospun polymer.

4. The tissue graft according to any one of the preceding claims, wherein said first scaffold comprises a melt-electrospun polymer, and said second scaffold comprises a solution-electrospun polymer.

5. The tissue graft according to any one of the preceding claims, wherein said average first pore size is from about 100μm – 1000μm in diameter.

6. The tissue graft according to any one of the preceding claims, wherein said first polymer fibers comprise fibers from about 10μm – 500μm in diameter.

7. The tissue graft according to any one of the preceding claims, wherein said average second pore size is from about 1 – 10μm in diameter.

8. The tissue graft according to any one of the preceding claims, wherein said first polymer fibers and/or said second polymer fibers comprise fibers from about 500nm – 10μm in diameter.

9. The tissue graft according to any one of the preceding claims, wherein said first polymeric scaffold is coated with calcium phosphate.

10. The tissue graft according to any one of the preceding claims, wherein said first polymeric scaffold and/or second polymeric scaffold is coated with one or more anti-inflammatory agents and/or one or more antibacterial agents.

11. The tissue graft according to claim 10, wherein an external surface of said first polymeric scaffold is coated with a membrane comprising said one or more anti-inflammatory agents and/or one or more antibacterial agents.

12. The tissue graft according to claim 11, wherein the membrane is a non-porous occlusive scaffold.

13. The tissue graft according to any one of claims 10 to 12, wherein said one or more antibacterial agents is also an anti-inflammatory agent.

14. The tissue graft according to any one of claims 10 to 12, wherein said one or more antibacterial agents is selected from the group consisting of macrolides, β-lactam antibiotics, metronidazole and tetracyclines.

15. The tissue graft according to any one of claims 10 to 13, wherein the antibacterial agent is a macrolide.

16. The tissue graft according to claim 15, wherein the antibacterial agent is azithromycin.

17. The tissue graft according to claim 16, wherein said azithromycin is present in an amount selected from any one of about 0.7 μg/mm^3 to about 3μg/mm^3, about 3μg/mm^3 to about 10μg/mm^3, about 10μg/mm^3 to about 20μg/mm^3, about 20μg to about 50μg/mm^3, about 50μg/mm^3 to about 100μg/mm^3, about 100μg/mm^3 to about 200μg, about 200 μg/mm^3 to about 300 μg/mm^3, about 300 μg/mm^3 to about 400 μg/mm^3, about 400 μg/mm^3 to about 500 μg/mm^3.

18. The tissue graft according to claim 16, wherein said azithromycin is present in an amount of about 3μg/mm^3 to about 100 μg/mm^3.

19. The tissue graft according to claim 16, wherein said azithromycin is present in an amount of about 20 μg/mm^3.

20. The tissue graft according to claim 16, wherein said azithromycin is present in an amount of about 10 μg /mm^3.

21. The tissue graft according to claim 16 wherein said azithromycin is present in an amount of about 5 μg /mm^3.

22. The tissue graft according to any one of the preceding claims, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of biodegradable polymers.

23. The tissue graft according to any one of claims 1 to 21, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of non-biodegradable polymers.

24. The tissue graft according to any one of claims 1 to 21, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of polymers selected from the group consisting of: aliphatic polyesters, poly(amino acids), modified proteins, polydepsipeptides, copoly(ether-esters), polyurethanes, polyalkylenes oxalates, polyamides, poly(iminocarbonates), polyorthoesters, polyoxaesters, polyamidoesters, poly(ε-caprolactone)s, polyanhydrides, polyarylates, polyphosphazenes, polyhydroxyalkanoates, polysaccharides, modified polysaccharides, polycarbonates, polytyrosinecarbonates, polyorthocarbonates, poly(trimethylene carbonate), poly(phosphoester)s, polyglycolide, polylactides,

polyhydroxybutyrates, polyhydroxyvalerates, polydioxanones, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(maleic anhydride), polyvinylalcohol, polyesteramides, polycyanoacrylates, polyfumarates, poly(ethylene glycol), polyoxaesters containing amine groups, poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(dioxanone)s, poly(alkylene alkylate)s, biopolymers, collagen, silk, chitosan, alginate, and a blend or copolymer of two or more of the preceding polymers.

25. The tissue graft according to claim 24, wherein the first polymer fibers and/or the second polymer fibers are polycaprolactones.

26. The tissue graft according to any one of the preceding claims, wherein the osteoblast-derived and/or the periodontal ligament cell-derived extracellular matrix comprises any one or more of collagen, elastin, reticulin, fibronectin, laminin, adhesive glycoproteins, glycosaminoglycans (GAG), proteoglycans, chemoattractants, cytokines, and/or growth factors.

27. The tissue graft according to any one of the preceding claims, wherein the osteoblast-derived extracellular matrix comprises any one of more of collagen, proteoglycans, glycoproteins, non-collagenous proteins, osteocalcin, osteopontin, bone sialoprotein, and any molecule that may be found within the native bone extracellular matrix.

28. The tissue graft according to any one of the preceding claims, wherein the periodontal ligament cell-derived extracellular matrix comprises one of more of collagen, proteoglycans, glycoproteins, non-collagenous proteins, osteocalcin, osteopontin, bone sialoprotein, periostin, sclerostin, cementum attachment protein, and any molecule that may be found within the native periodontal ligament or cementum extracellular matrix.

29. A method of producing a decellularized biphasic periodontal tissue graft comprising:

adjoining a first polymeric scaffold comprising a plurality of first polymer fibers and having a porous structure with an average first pore size to a second polymeric scaffold comprising a plurality of second polymer fibers and having a porous structure with an average second pore size;

seeding said first scaffold with one or more osteoblasts and culturing the tissue graft in a tissue culture medium;

seeding said second scaffold with one or more layers of a periodontal ligament cell layer and culturing the tissue graft in a tissue culture medium; and

decellularizing the tissue graft after said seeding and culturing of the first and/or second polymeric scaffolds.

30. A method of producing a decellularized biphasic periodontal tissue graft comprising:

laying a plurality of first polymer fibers on a surface to provide a first polymeric scaffold having a porous structure with an average first pore size;

laying a plurality of second polymer fibers on a surface to provide a second polymeric scaffold having a porous structure with an average second pore size;

adjoining said first and said second polymeric scaffolds;

seeding said first scaffold with one or more osteoblasts and culturing the tissue graft in a tissue culture medium;

seeding said second scaffold with one or more layers of a periodontal ligament cell layer and culturing the tissue graft in a tissue culture medium; and

decellularizing the tissue graft after said seeding and culturing of the first and/or second polymeric scaffolds.

31. A method of producing a decellularized biphasic periodontal tissue graft comprising:

laying a plurality of first polymer fibers on a surface to provide a first polymeric scaffold having a porous structure with an average first pore size;

laying a plurality of second polymer fibers on said first polymeric scaffold to provide a second polymeric scaffold having a porous structure with an average second pore size, connected to said first polymeric scaffold;

seeding said first scaffold with one or more osteoblasts and culturing the tissue graft in a tissue culture medium;

seeding said second scaffold with one or more layers of a periodontal ligament cell layer and culturing the tissue graft in a tissue culture medium; and

decellularizing the tissue graft after said seeding and culturing of the first and/or second polymeric scaffolds.

32. A method of producing a decellularized biphasic periodontal tissue graft comprising: laying a plurality of first polymer fibers on a surface to provide a first polymeric scaffold having a porous structure with an average first pore size; laying a plurality of second polymer fibers on a surface to provide a second polymeric scaffold having a porous structure with an average second pore size; adjoining said first and said second polymeric scaffolds; seeding said first scaffold with one or more osteoblast and culturing the tissue graft in a tissue culture medium; harvesting one or more layers of a periodontal ligament cell layer previously cultured in a tissue culture medium with said second scaffold; and decellularizing the tissue graft after said seeding and culturing of the first and/or second polymeric scaffolds

33. The method according to any one of claims 29 to 32, wherein said laying of the first and/or said second polymer fibers is by electrospinning.

34. The method according to any one of claims 29 to 33, wherein said average first pore size is greater than said average second pore size.

35. The method according to any one of claims 29 to 33, wherein said average first pore size is from about 100μm – 1000 μm in diameter.

36. The method according to any one of claims 29 to 35, wherein said average second pore size is from about 1μm – 10μm in diameter.

37. The method according to any one of claims 29 to 35, wherein first and/or second polymer fibers comprise fibers from about 500 nm– 10μm in diameter.

38. The method according to any one of claims 29 to 37, further comprising the step of coating said first polymeric scaffold with calcium phosphate prior to seeding the first polymeric scaffold with osteoblasts.

39. The method according to any one of claims 29 to 38, further comprising the step of coating said first and/or second polymeric scaffold with one or more anti-inflammatory agents and/or one or more antibacterial agents.

40. The method according to claim 39, wherein said coating is performed prior to seeding the first polymeric scaffold with osteoblasts.

41. The method according to claim 40, wherein said coating comprises applying to an external surface of said first polymeric scaffold a membrane comprising said one or more anti-inflammatory agents and/or one or more antibacterial agents.

42. The method according to claim 41, wherein the membrane is a non-porous occlusive scaffold.

43. The method according to any one of claims 39 to 42, wherein said one or more antibacterial agents is also an anti-inflammatory agent.

44. The method according to any one of claims 39 to 42, wherein said one or more antibacterial agents is selected from the group consisting of, macrolides, β-lactam antibiotics, metronidazole and tetracyclines.

45. The method according to any one of claims 39 to 44, wherein said antibacterial agent is a macrolide.

46. The method according to claim 45, wherein said antibacterial agent is azithromycin.

47. The method according to claim 46, wherein said azithromycin is present in an amount selected from any one of about 0.7μg/mm^3 to about 3μg/mm^3, about 3μg/mm^3 to about 10μg/mm^3, about 10μg/mm^3 to about 20μg/mm^3, about 20μg to about 50μg/mm^3, about 50μg/mm^3 to about 100μg/mm^3, about 100μg/mm^3 to about 200μg, about 200μg/mm^3 to about 300μg/mm^3, about 300μg/mm^3 to about 400μg/mm^3, about 400μg/mm^3 to about 500μg/mm^3.

48. The method according to claim 46, wherein said azithromycin is present in an amount of about 3μg/mm^3 to about 100μg/mm^3.

49. The method according to claim 46, wherein azithromycin is present in an amount of about 20μg/mm^3.

50. The method according to claim 46, wherein said azithromycin is present in an amount of about 10μg/mm^3.

51. The method according to claim 46, wherein said azithromycin is present in an amount of about 5μg/mm^3

52. The method according to any one of claims 39, 40 or 42 to 51, wherein the scaffold is coated with said agent by:

dissolving an amount of said agent in ethanol and applying to the tissue graft;
incubating the graft for a suitable time period;
evaporating the ethanol at room temperature; and
washing the grafts after the evaporation of ethanol.

53. The method according to any one of claims 29 to 52, wherein said decellularizing comprises the steps of perfusing the tissue graft with a solution of NH_4OH solution and Triton X-100, perfusing the graft with a DNase I solution, and perfusing the graft with sterile water.

54. The method according to claim 53, wherein each perfusion step is performed for about 30 minutes to 2 hours.

55. The method according to claim 53 or 54, wherein the perfusion is bi-directional.

56. The method according to any one of claims 28 to 55, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of biodegradable polymers.

57. The method according to any one of claims 28 to 55, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of non-biodegradable polymers

58. The method according to any one of claims 28 to 55, wherein the first polymer fibers and/or the second polymer fibers comprise or consist of polymers selected from the group consisting of: aliphatic polyesters, poly(amino acids), modified proteins, polydepsipeptides, copoly(ether-esters), polyurethanes, polyalkylenes oxalates, polyamides, poly(iminocarbonates), polyorthoesters, polyoxaesters, polyamidoesters, poly(ε-caprolactone)s, polyanhydrides,

polyarylates, polyphosphazenes, polyhydroxyalkanoates, polysaccharides, modified polysaccharides, polycarbonates, polytyrosinecarbonates, polyorthocarbonates, poly(trimethylene carbonate), poly(phosphoester)s, polyglycolide, polylactides, polyhydroxybutyrates, polyhydroxyvalerates, polydioxanones, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(maleic anhydride), polyvinylalcohol, polyesteramides, polycyanoacrylates, polyfumarates, poly(ethylene glycol), polyoxaesters containing amine groups, poly(lactide-co-glycolides), poly(lactic acid)s, poly(glycolic acid)s, poly(dioxanone)s, poly(alkylene alkylate)s, biopolymers, collagen, silk, chitosan, alginate, and a blend or copolymer of two or more of the preceding polymers.

59. The method according to claim 58, wherein the first polymer fibers and/or the second polymers are polycaprolactones.

60. The method according to any one of claims 28 to 59, further comprising a step of sterilizing the tissue graft.

61. The method according to claim 60, wherein said sterilization is via ethlylene oxide, UV irradiation or gamma irradiation.

62. A decellularized biphasic periodontal tissue graft obtained or obtainable by the method according to any one of claims 29 to 61.

63. A method of treating and/or repairing a periodontal defect in a subject in need thereof, comprising implanting a tissue graft according to any one of claims 1 to 28 into the periodontium of said subject.

64. A method of treating periodontal disease or degeneration in a subject in need thereof, comprising implanting a tissue graft according to any one of claims 1 to 28 into the periodontium of said subject.

65. A method of regenerating periodontal tissue in a subject comprising implanting a tissue graft according to any one of claims 1 to 28 into the periodontium of said subject.

66. A composition according to any one of claims 1 to 28 for use in treating and/or repairing a periodontal defect in a subject in need thereof.

67. A composition according to any one of claims 1 to 28 for use in treating periodontal disease in a subject in need thereof.

68. A composition according to any one of claims 1 to 28 for use in regenerating periodontal tissue in a subject.

69. Use of a composition according to any one of claims 1 to 28 in the manufacture of a medicament for treating and/or repairing a periodontal defect in a subject.

70. Use of a composition according to any one of claims 1 to 28 in the manufacture of a medicament for treating periodontal disease in a subject.

71. Use of a composition according to any one of claims 1 to 28 in the manufacture of a medicament for regenerating periodontal tissue in a subject.

72. The method according to any one of claims 63 to 65, the composition according to any one of claims 66 to 68, or the use according to any one of claims 69 to 71, wherein the subject is a human subject.

73. The method according to any one of claims 63 to 66, wherein implanting a tissue graft comprises excising a portion of the gingival tissue of the subject to access bone and tooth root surface within said periodontium and placing said tissue graft on a surface of said bone and tooth root surface.

Griffith University

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

FIGURE 1

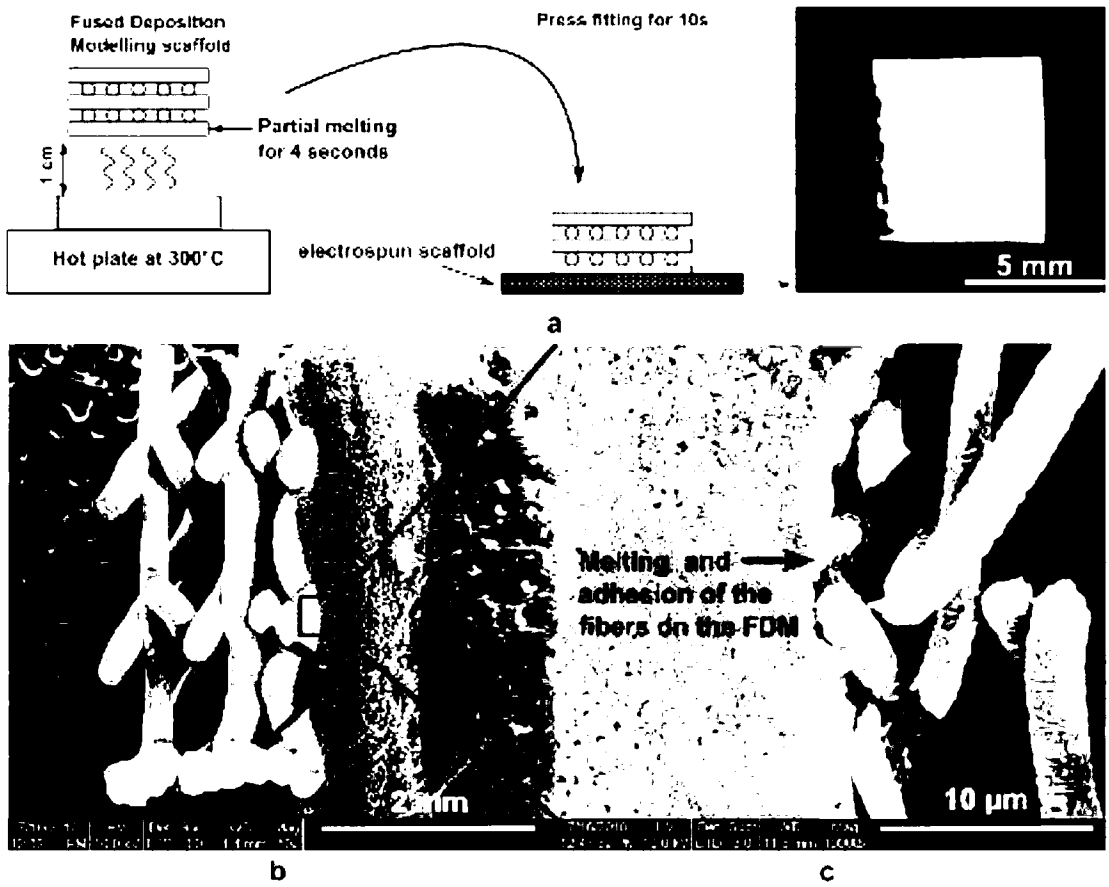

Fused Deposition Modelling scaffold
Press fitting for 10s
Partial melting for 4 seconds
1 cm
Hot plate at 300°C
electrospun scaffold
5 mm
a
Melting and adhesion of the fibers on the FDM
2 µm
10 µm
b
c

FIGURE 1 CONT'D

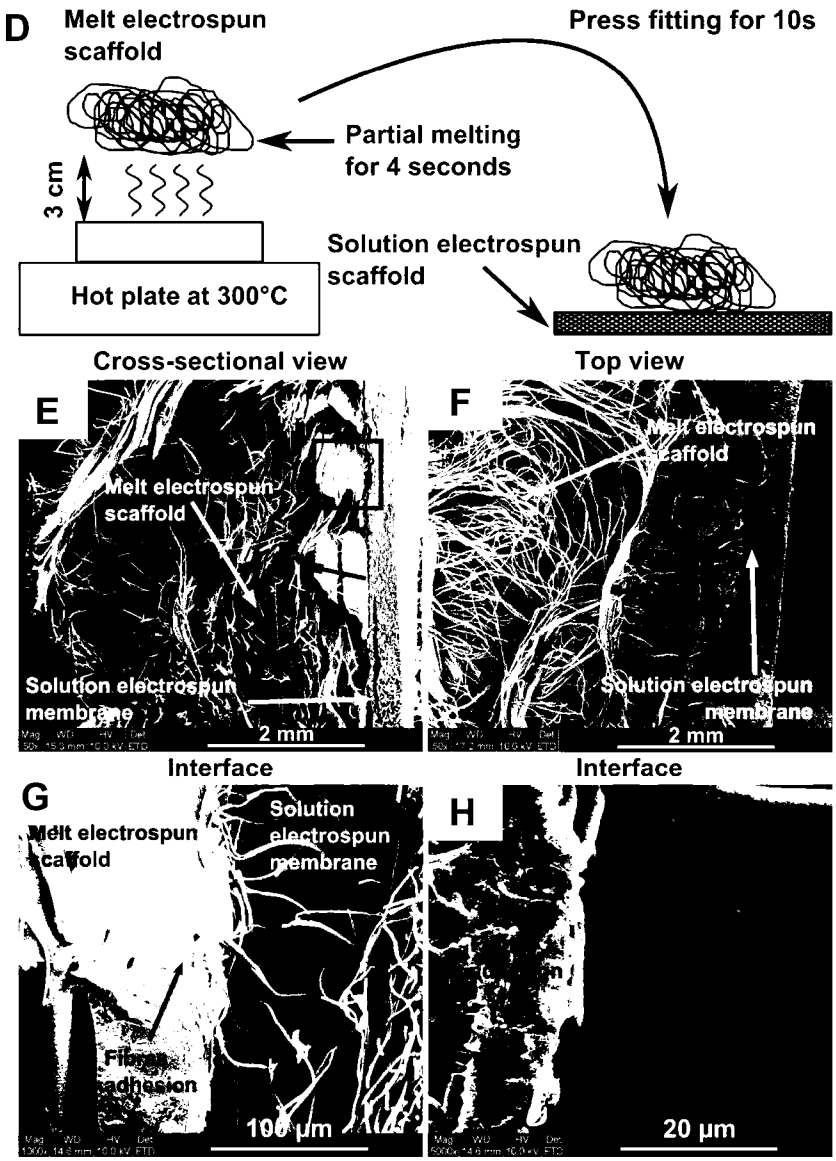


FIGURE 2

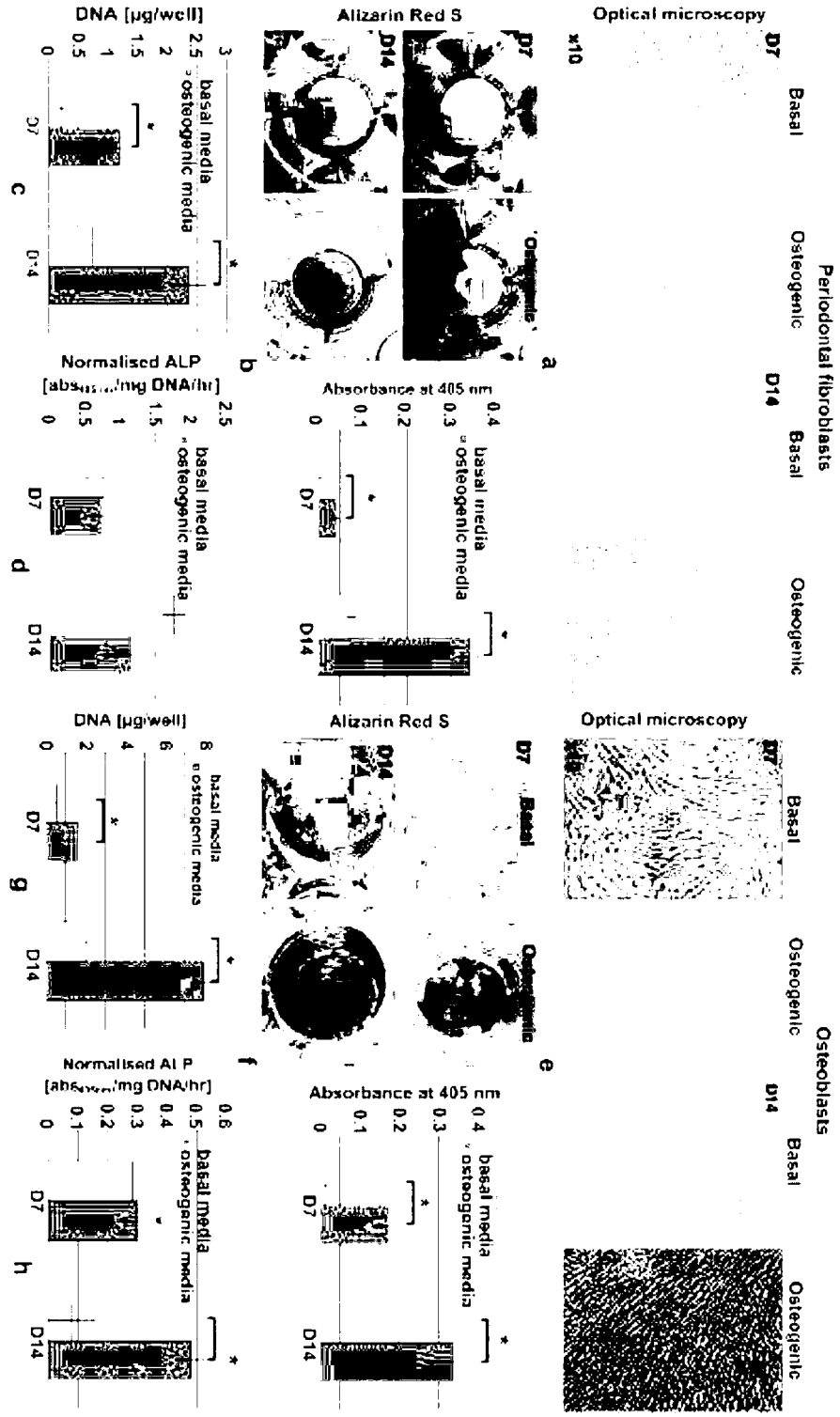

Optical microscopy
Alizarin Red S
DNA [µg/well]
Normalised ALP
Absorbance at 405 nm
Periodontal fibroblasts
Osteoblasts
Basal
Osteogenic
D7
D14
basal media
osteogenic media
a
b
c
d
e
f
g
h

FIGURE 3

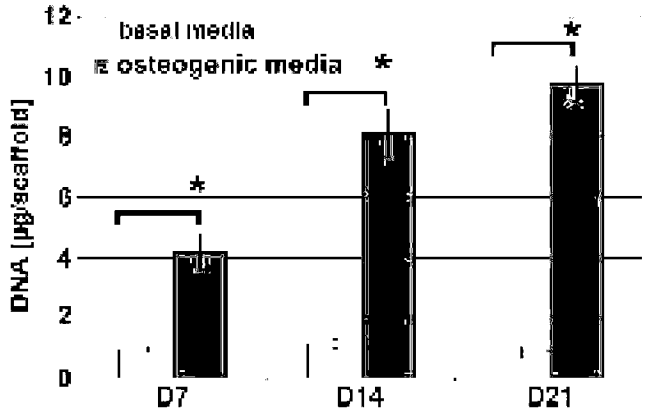


Figure 4

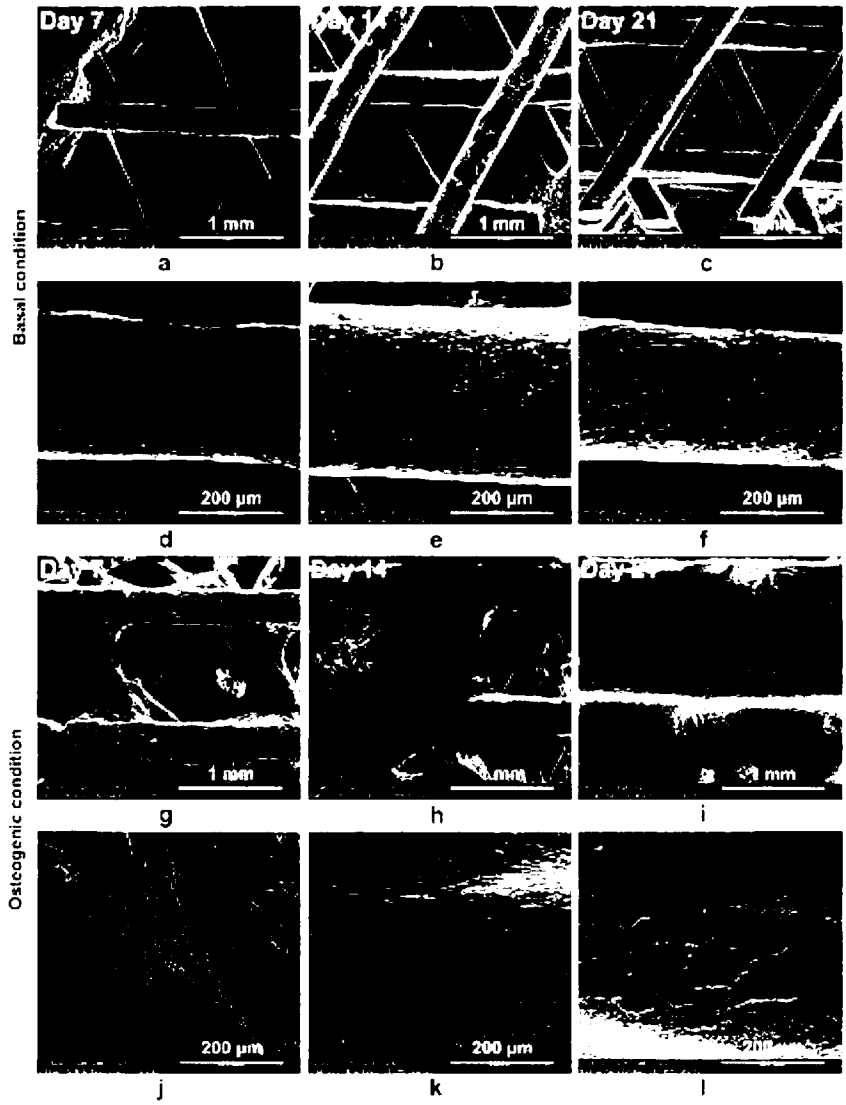


FIGURE 5

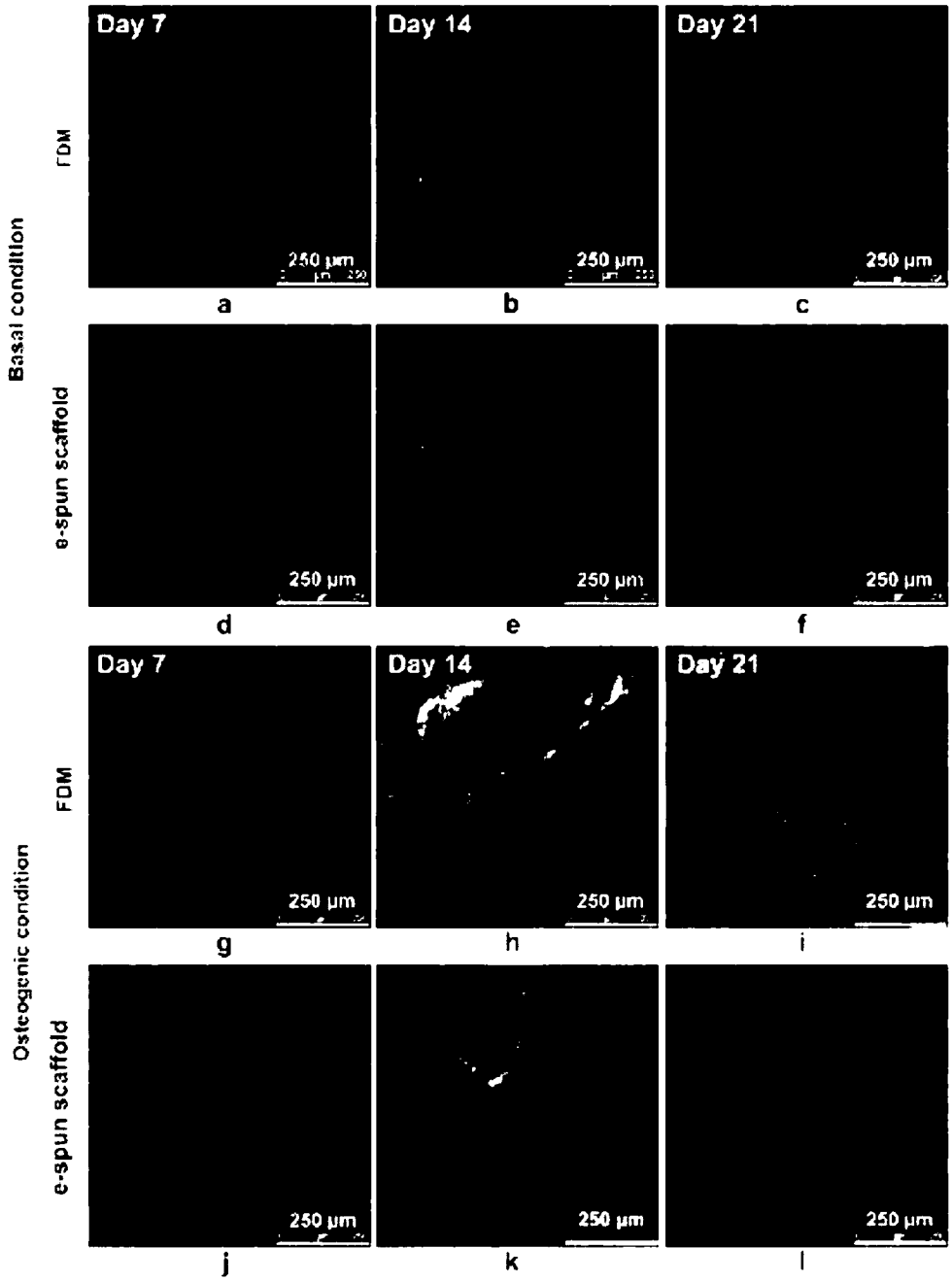

Day 7
Day 14
Day 21
FDM
Basal condition
e-spun scaffold
250 µm
a
b
c
d
e
f
Day 7
Day 14
Day 21
FDM
Osteogenic condition
e-spun scaffold
250 µm
g
h
i
j
k
l

FIGURE 6

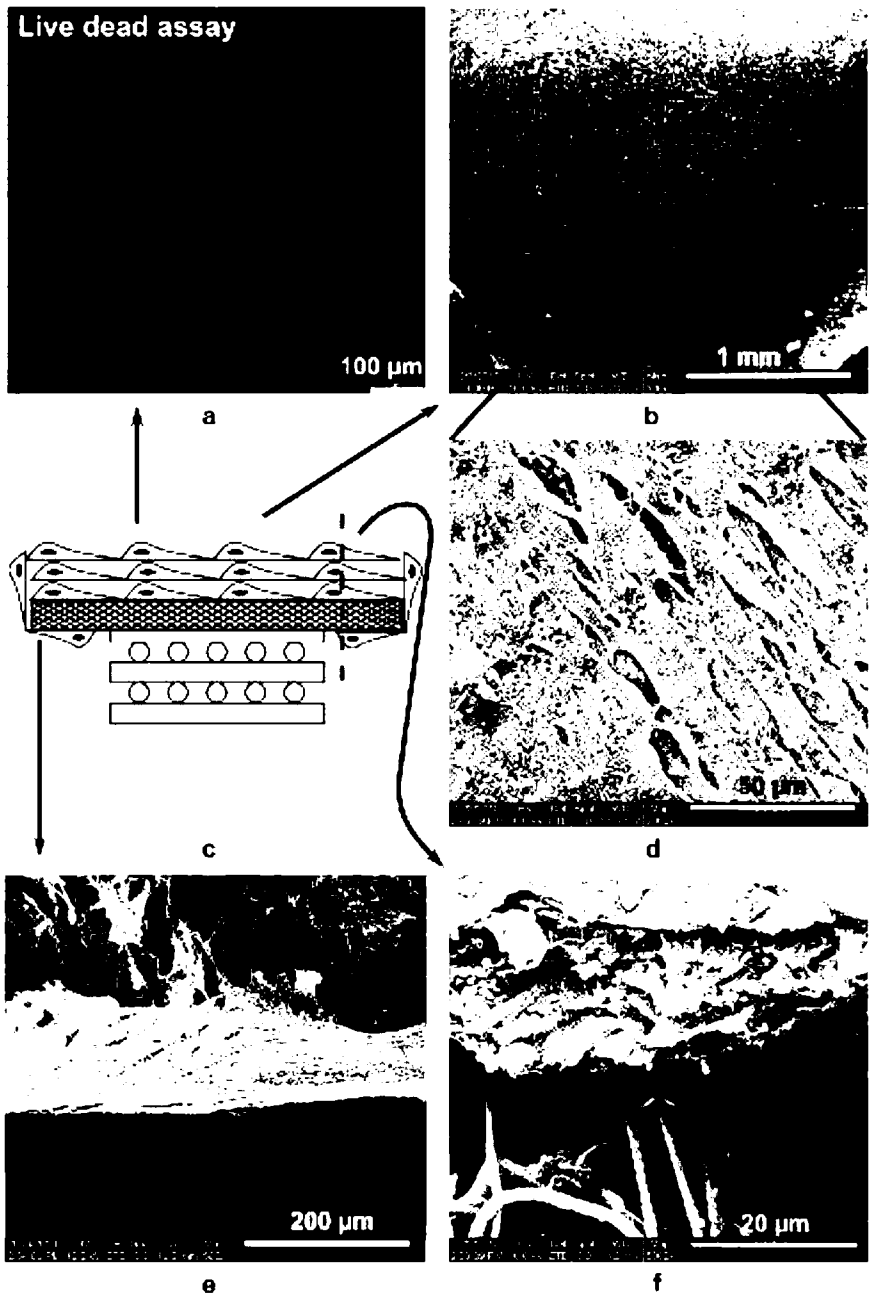


FIGURE 7

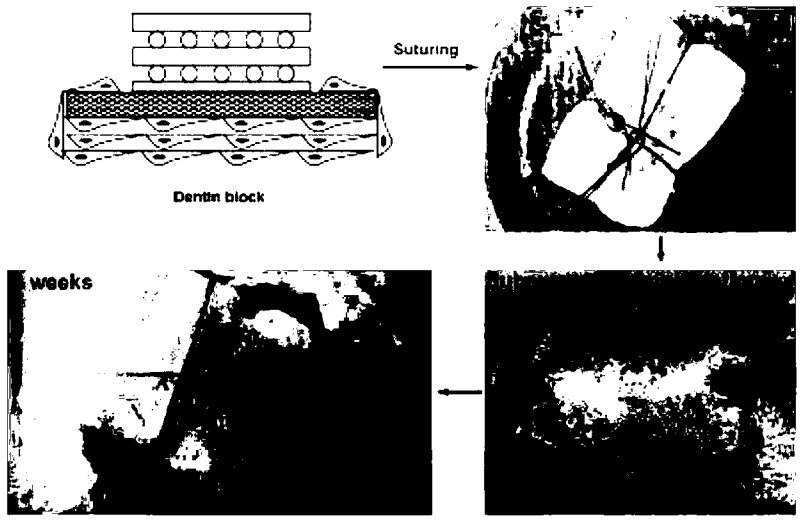


Figure 8

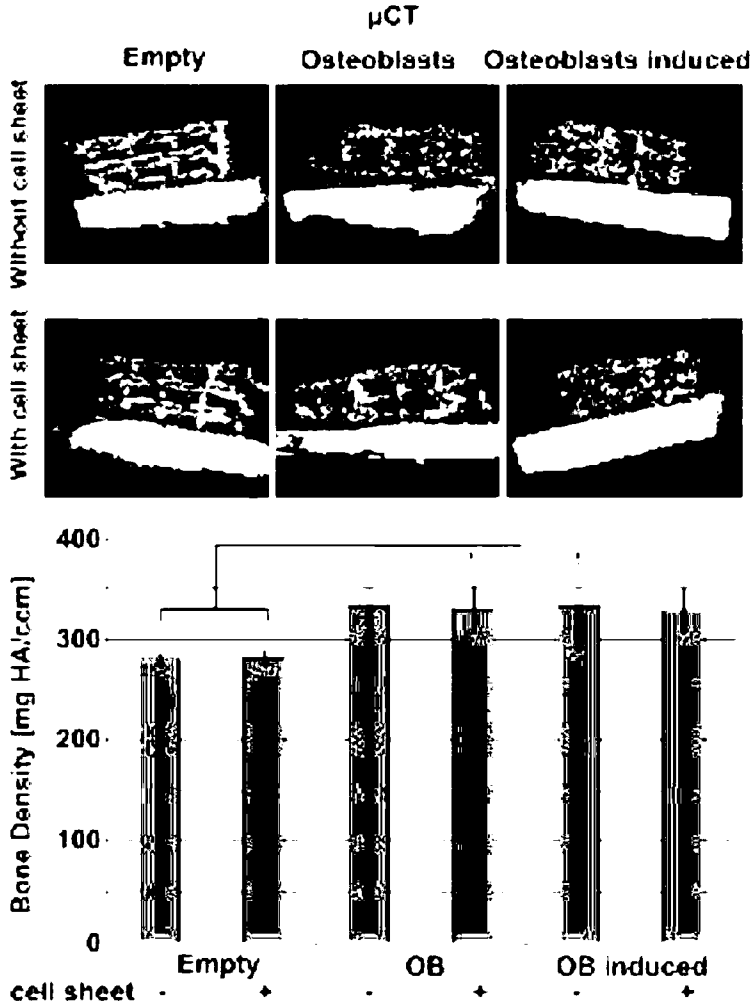

µCT
Empty
Osteoblasts
Osteoblasts induced
Without cell sheet
With cell sheet
Bone Density [mg HA/ccm]
400
300
200
100
0
Empty
OB
OB induced
cell sheet - + - + - +

Figure 9

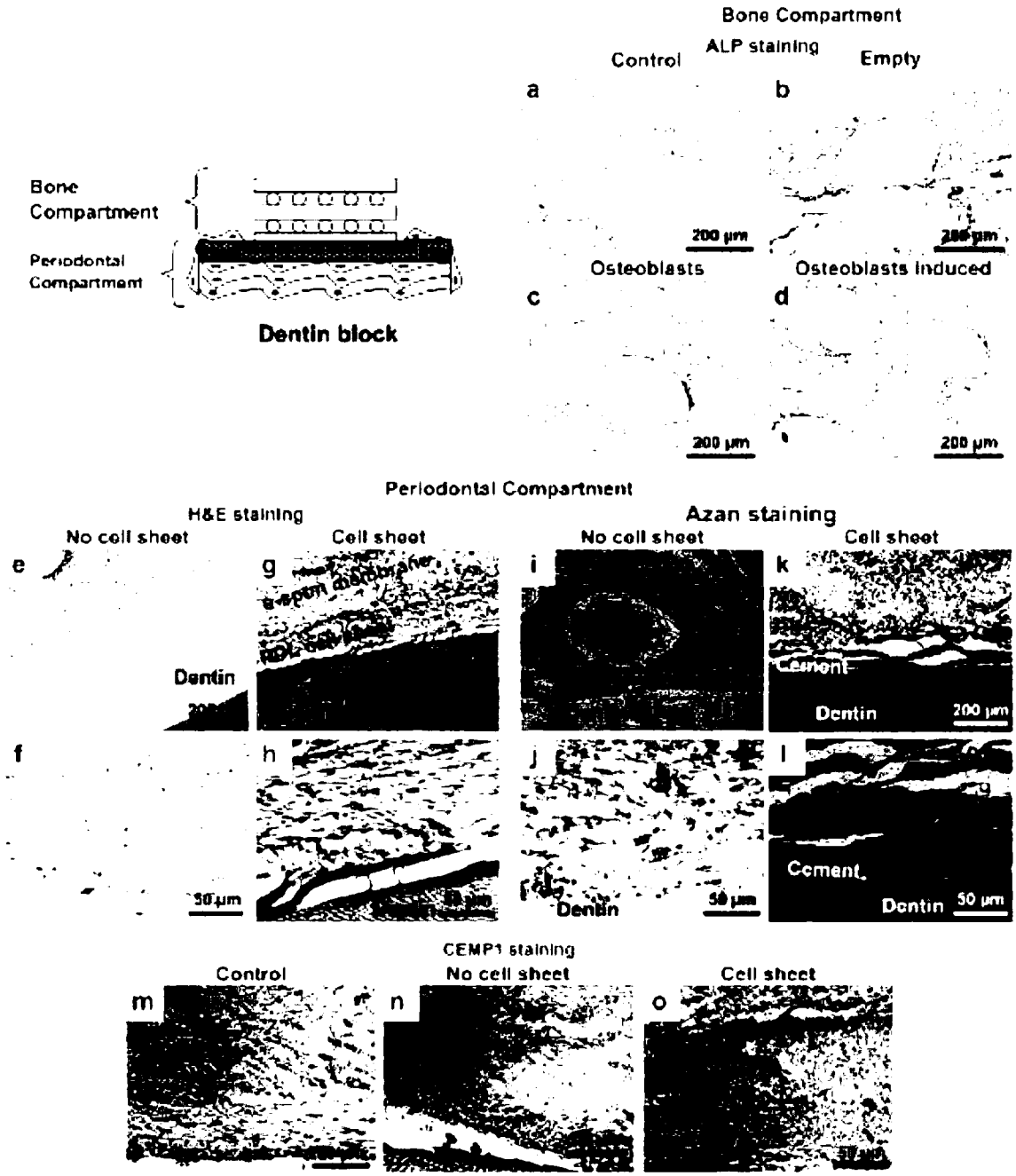

Bone Compartment
Periodontal Compartment
Dentin block
Bone Compartment
ALP staining
Control
Empty
a
b
200 µm
Osteoblasts
Osteoblasts induced
c
d
200 µm
Periodontal Compartment
H&E staining
Azan staining
No cell sheet
Cell sheet
No cell sheet
Cell sheet
e
g
i
k
Dentin
Cement
Dentin
200 µm
f
h
j
l
50 µm
Dentin
Cement
Dentin
50 µm
CEMP1 staining
Control
No cell sheet
Cell sheet
m
n
o

FIGURE 10

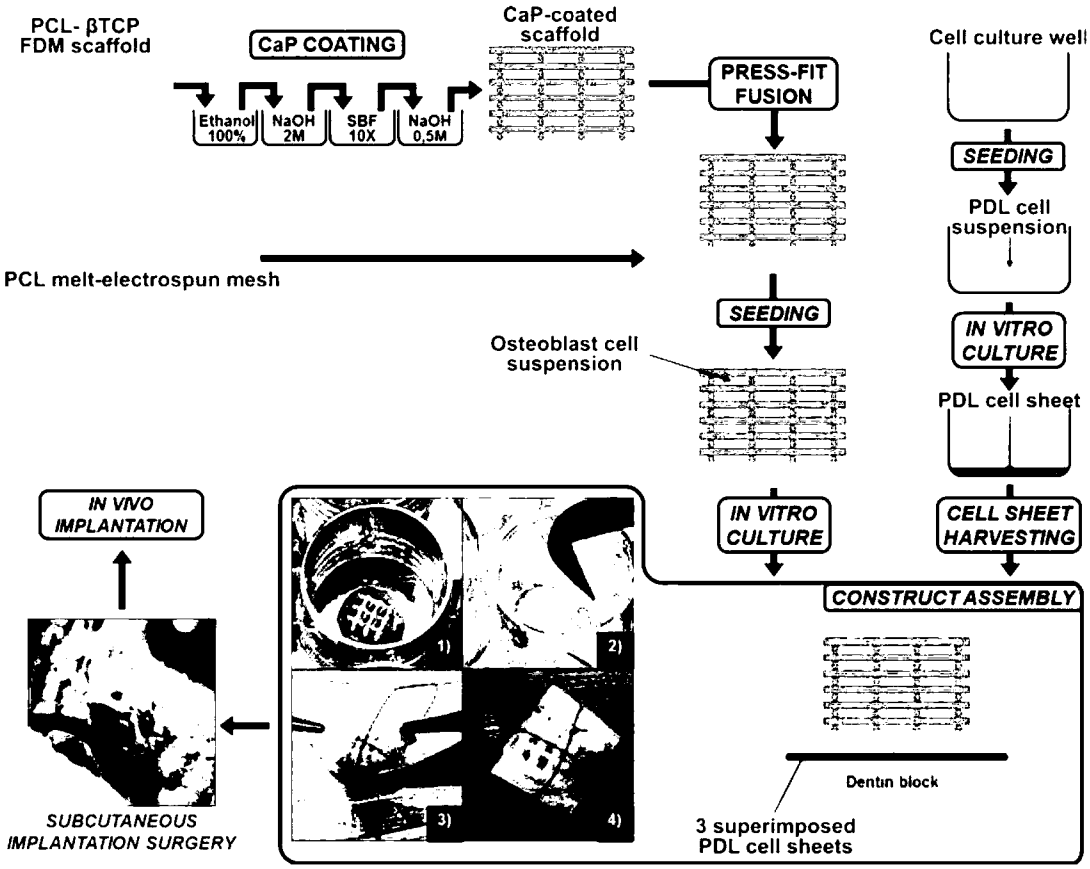

PCL- βTCP
FDM scaffold
CaP COATING
Ethanol 100%
NaOH 2M
SBF 10X
NaOH 0,5M
CaP-coated scaffold
PRESS-FIT FUSION
Cell culture well
SEEDING
PDL cell suspension
PCL melt-electrospun mesh
SEEDING
Osteoblast cell suspension
IN VITRO CULTURE
PDL cell sheet
IN VIVO IMPLANTATION
IN VITRO CULTURE
CELL SHEET HARVESTING
CONSTRUCT ASSEMBLY
1)
2)
3)
4)
Dentin block
3 superimposed PDL cell sheets
SUBCUTANEOUS IMPLANTATION SURGERY

FIGURE 11

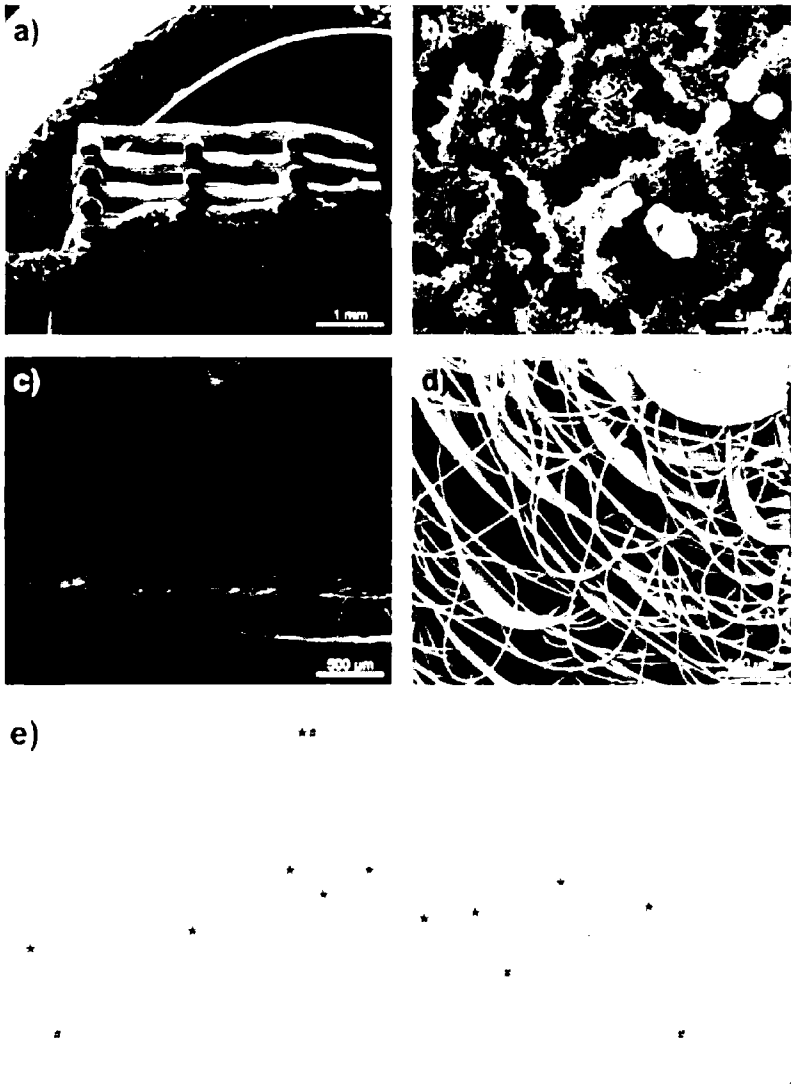


FIGURE 12

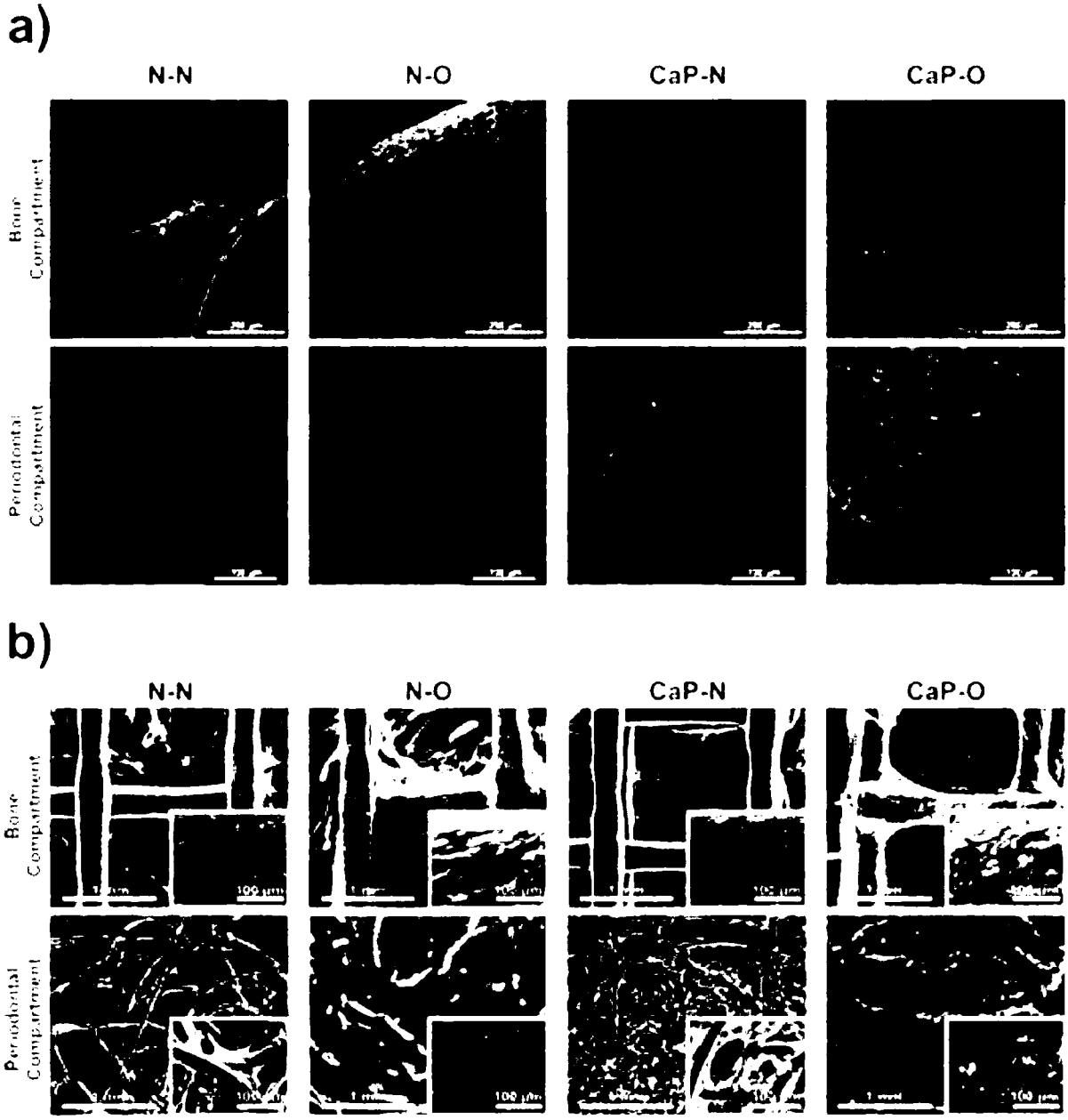

a)
N-N
N-O
CaP-N
CaP-O
Bone Compartment
Periodontal Compartment
b)
N-N
N-O
CaP-N
CaP-O
Bone Compartment
Periodontal Compartment

FIGURE 13

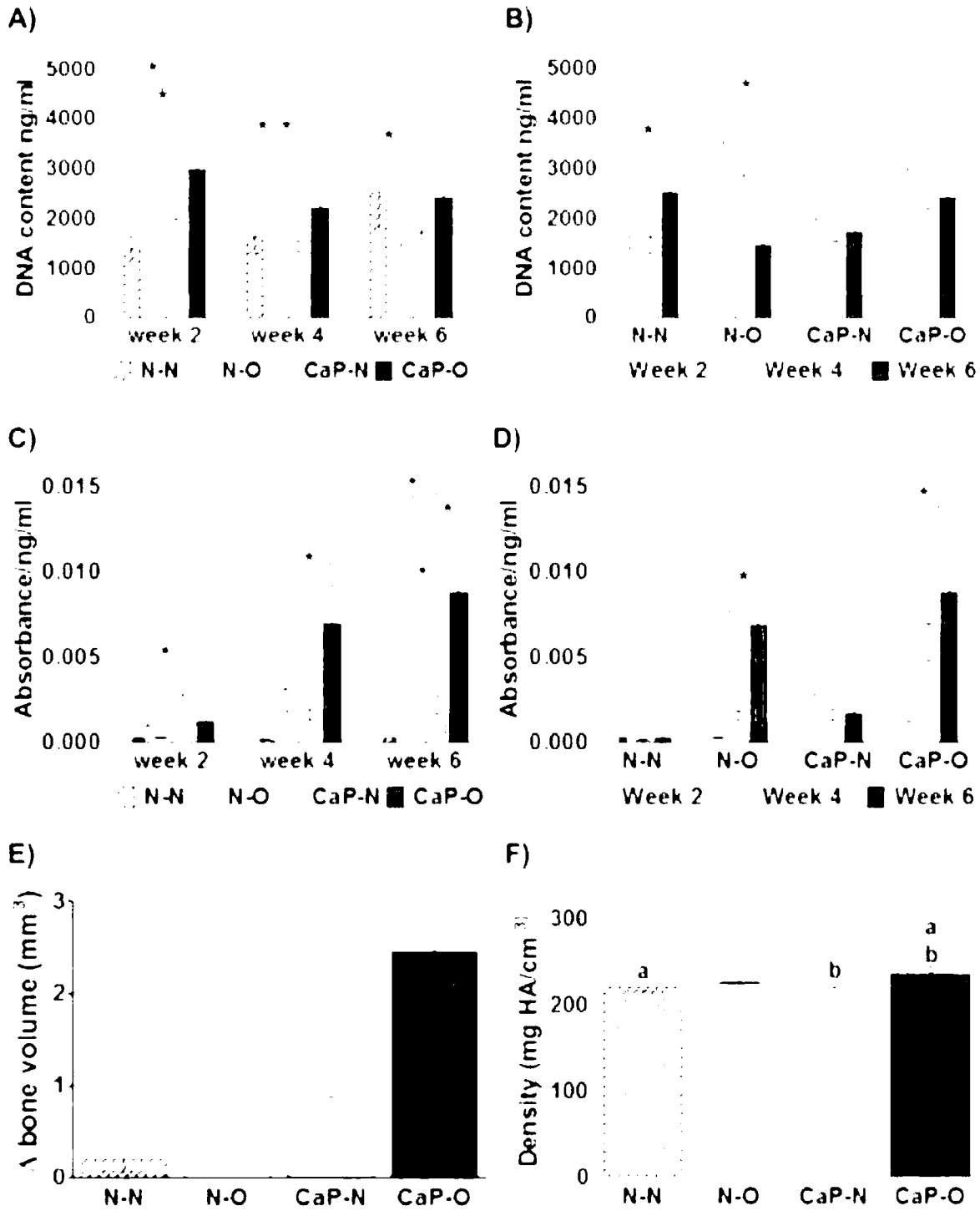

A)
DNA content ng/ml
5000
4000
3000
2000
1000
0
week 2
week 4
week 6
N-N
N-O
CaP-N
CaP-O
B)
DNA content ng/ml
5000
4000
3000
2000
1000
0
N-N
N-O
CaP-N
CaP-O
Week 2
Week 4
Week 6
C)
Absorbance/ng/ml
0.015
0.010
0.005
0.000
week 2
week 4
week 6
N-N
N-O
CaP-N
CaP-O
D)
Absorbance/ng/ml
0.015
0.010
0.005
0.000
N-N
N-O
CaP-N
CaP-O
Week 2
Week 4
Week 6
E)
bone volume (mm³)
3
2
1
0
N-N
N-O
CaP-N
CaP-O
F)
Density (mg HA/cm³)
300
200
100
0
a
b
a
b
N-N
N-O
CaP-N
CaP-O

FIGURE 14

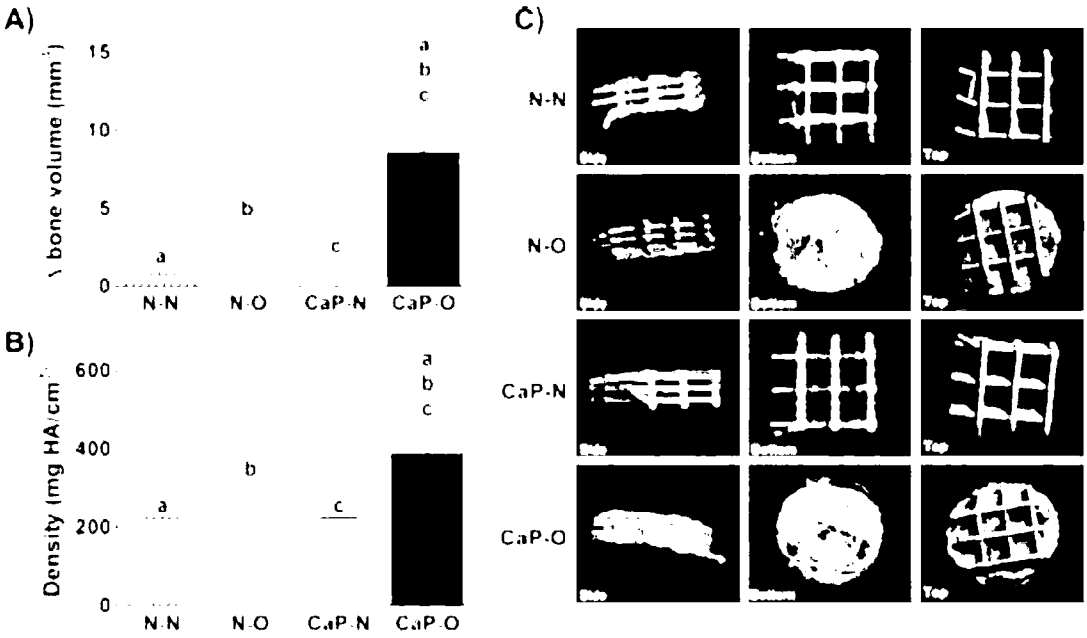


FIGURE 15

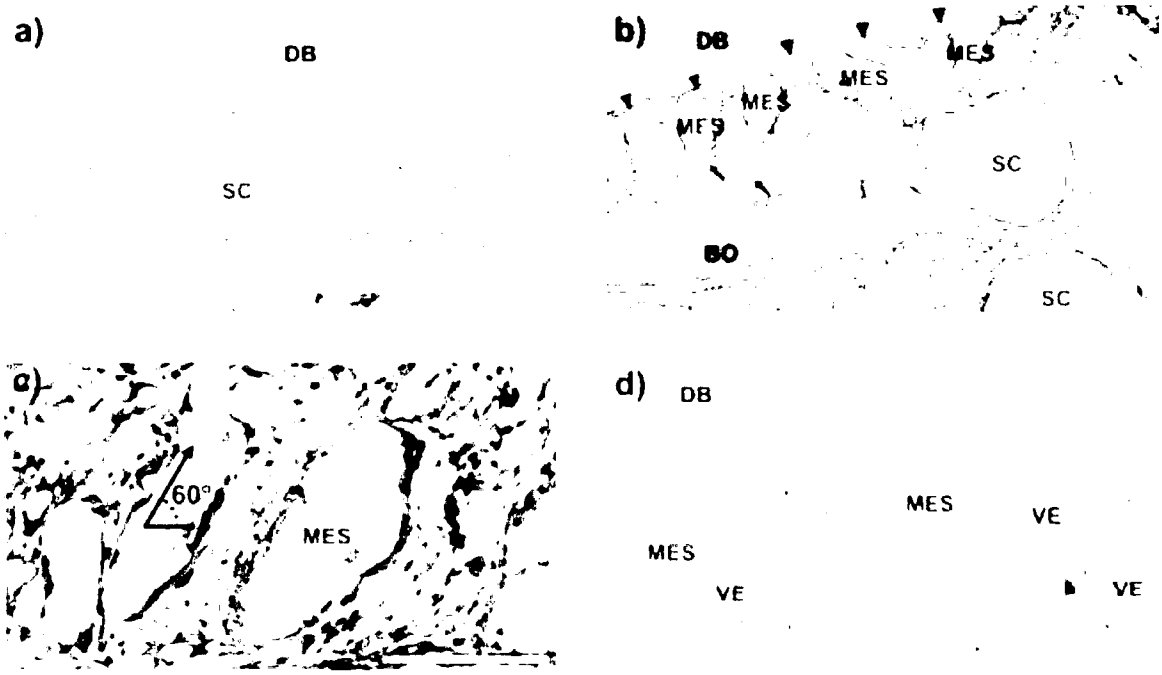


Figure 16

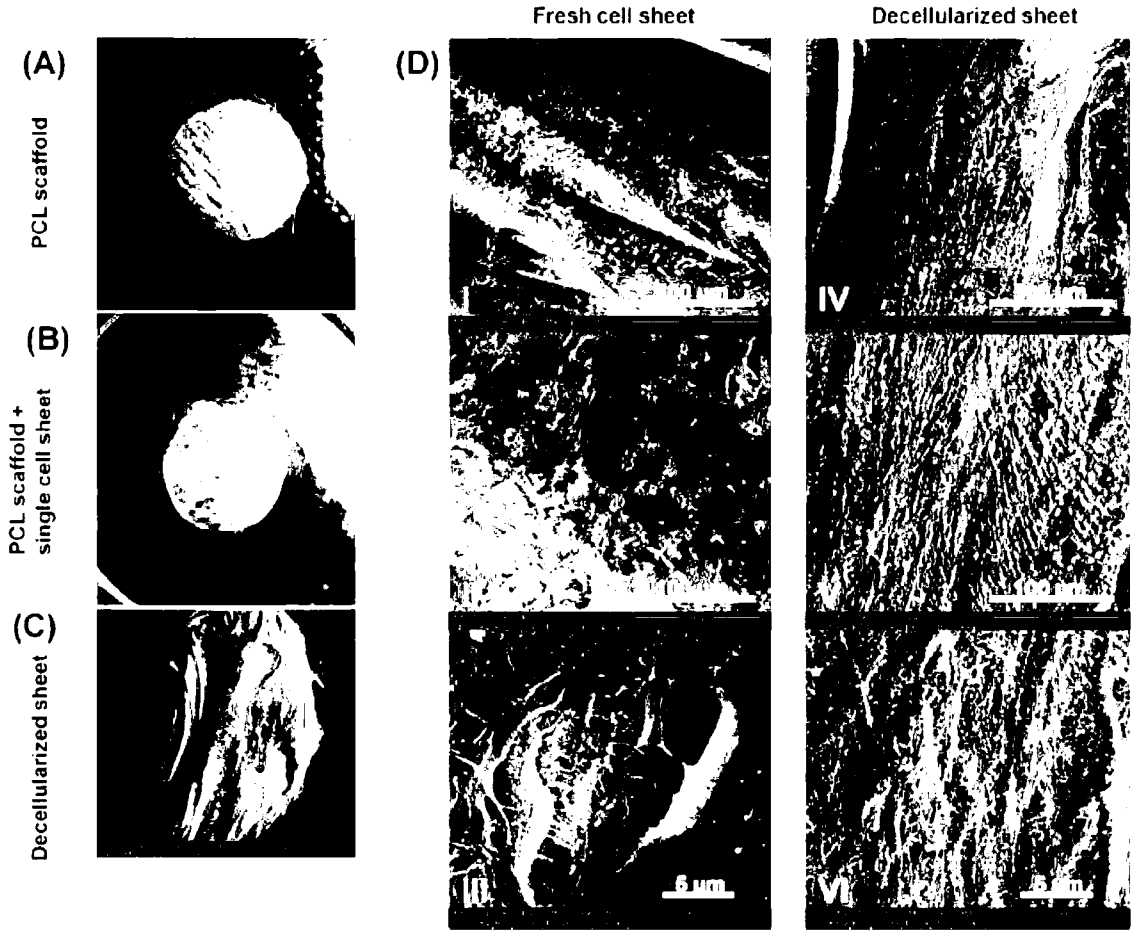

(A)
PCL scaffold
(B)
PCL scaffold + single cell sheet
(C)
Decellularized sheet
(D)
Fresh cell sheet
Decellularized sheet
IV
III
VI
5 μm

Figure 17

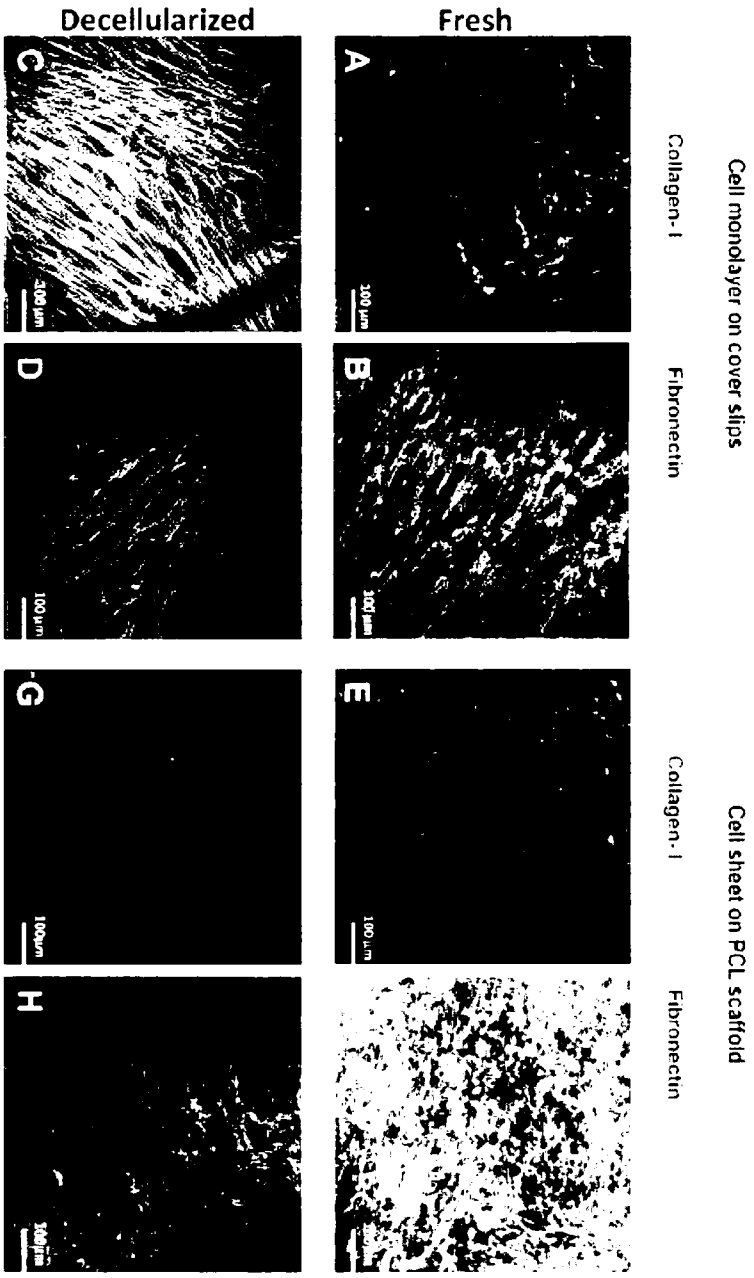

Decellularized
Fresh
C
A
D
B
G
E
H
F
Collagen-I
Fibronectin
Collagen-I
Fibronectin
Cell monolayer on cover slips
Cell sheet on PCL scaffold

Figure 18

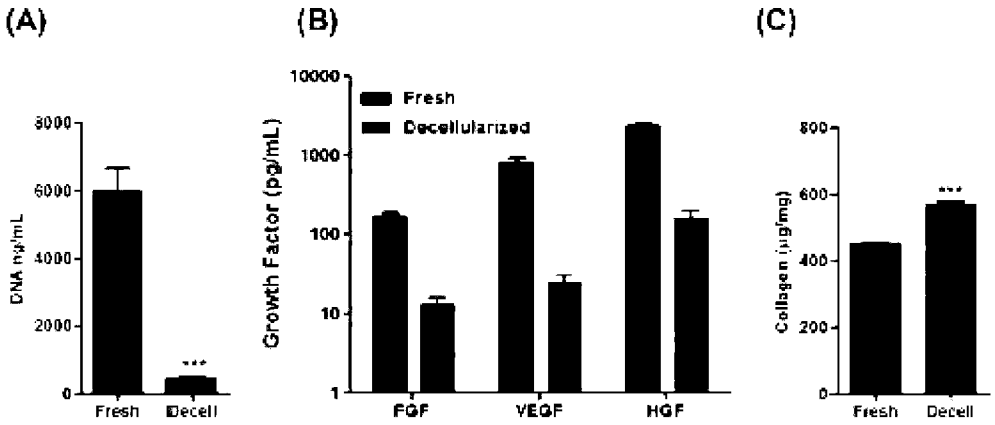


Figure 19

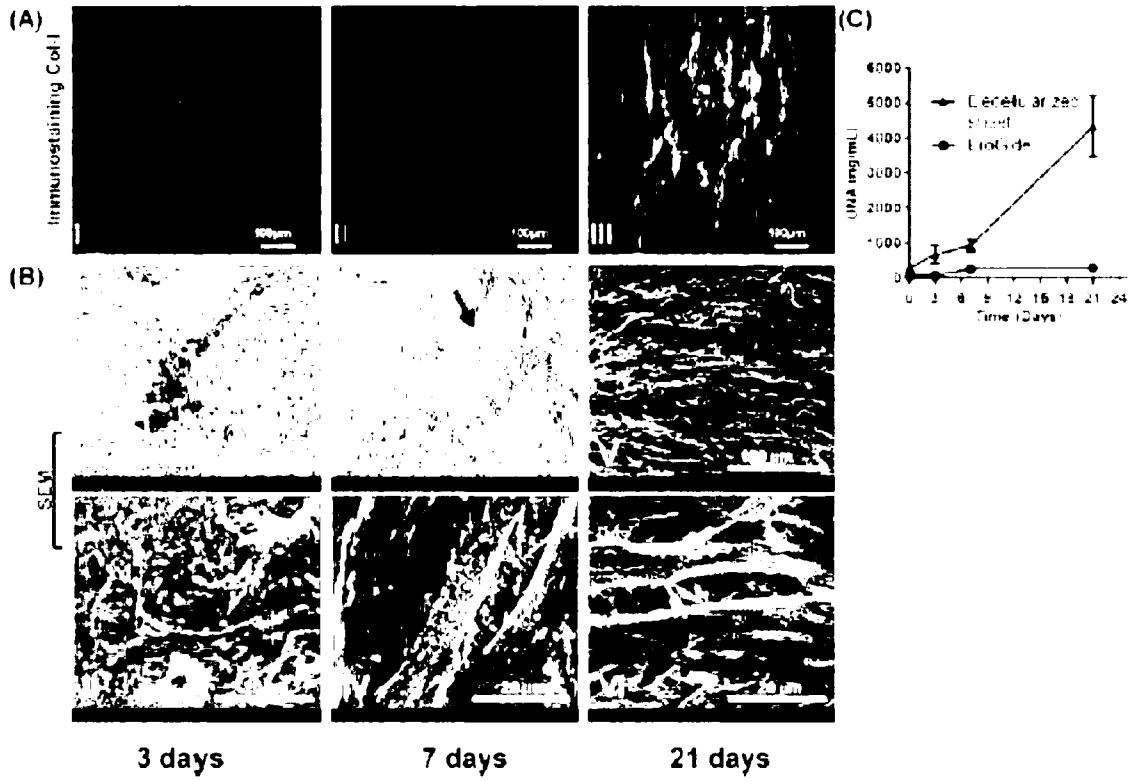


Figure 20

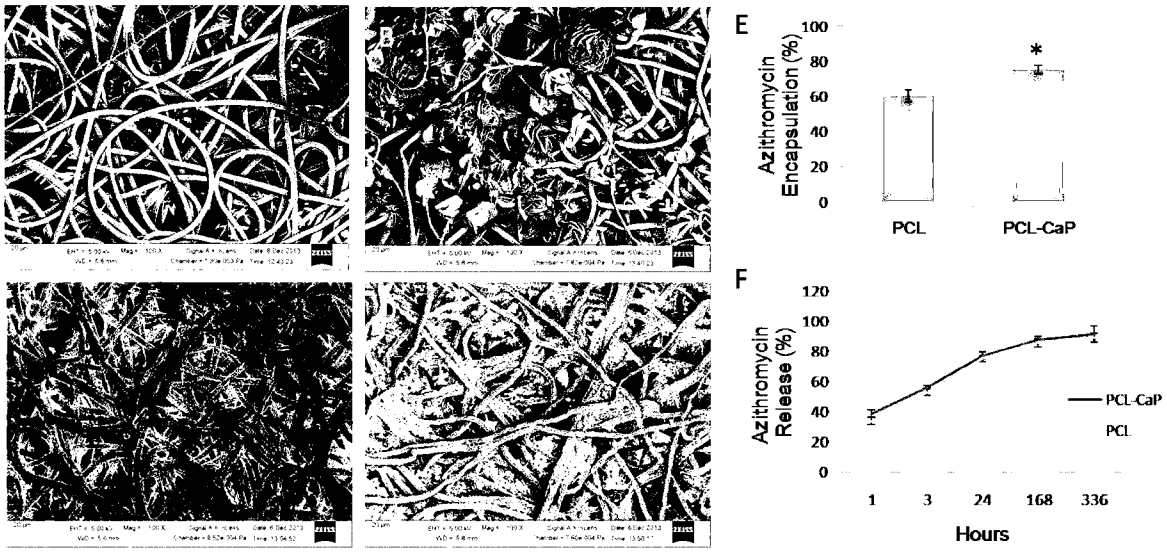


Figure 21

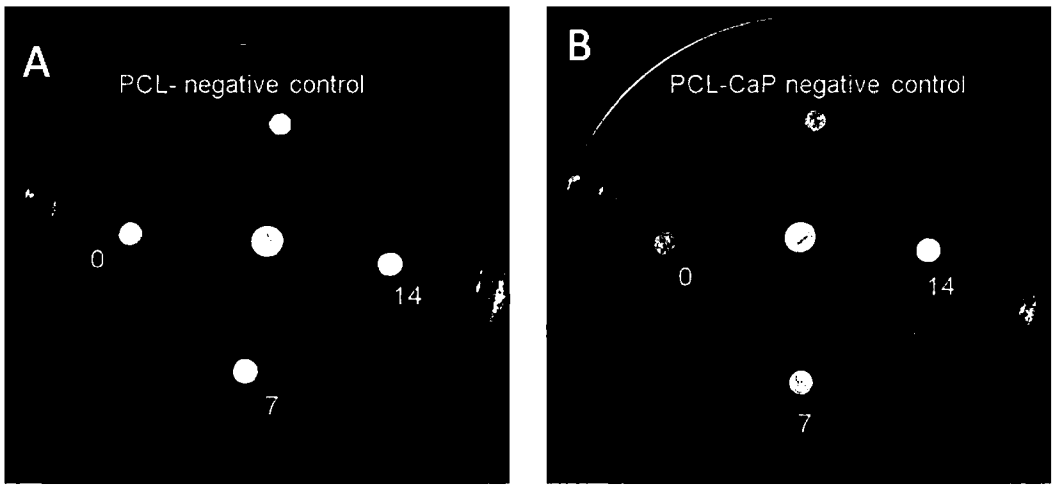
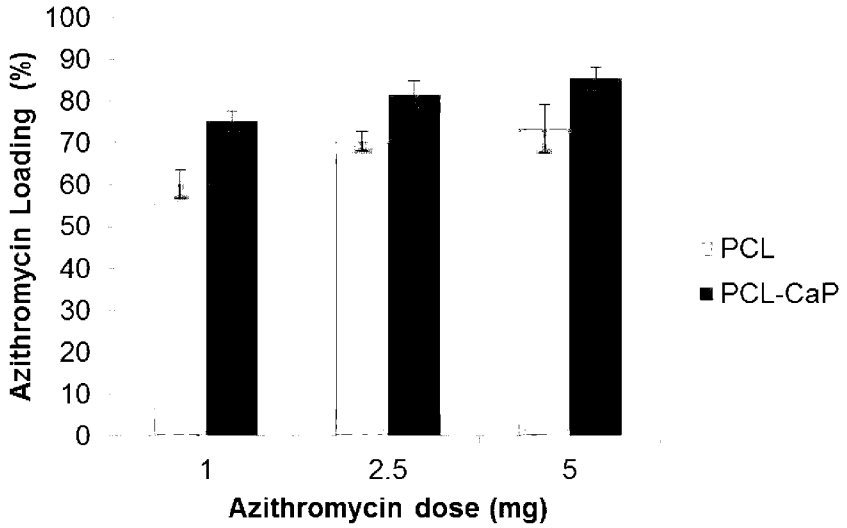


FIGURE 22

High Dose



(A)

(B)

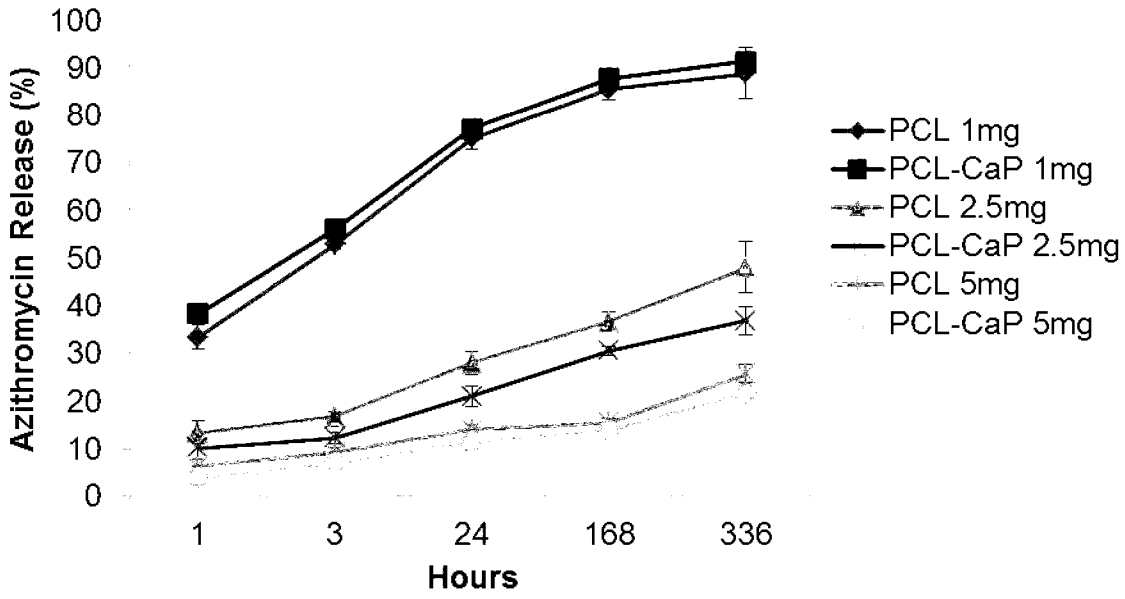
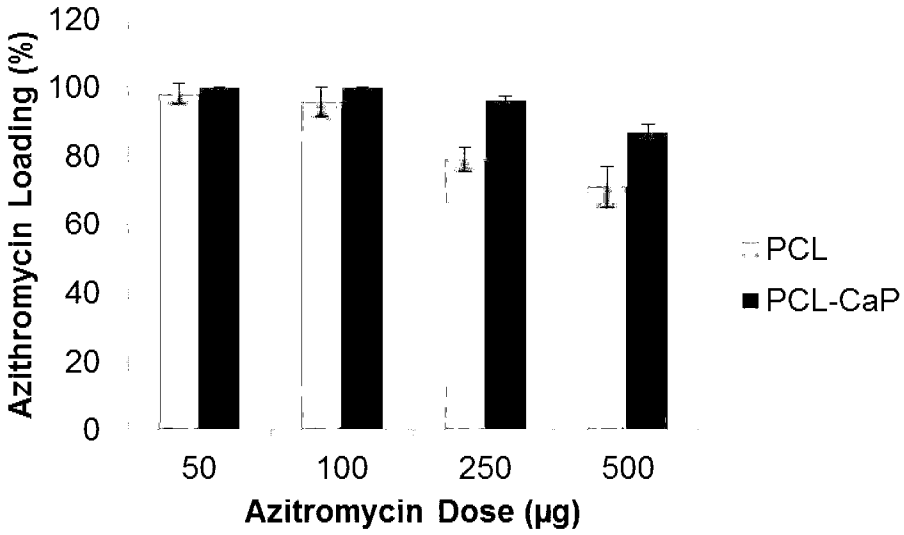


FIGURE 22 CONT'D

Low Dose



(C)

(D)

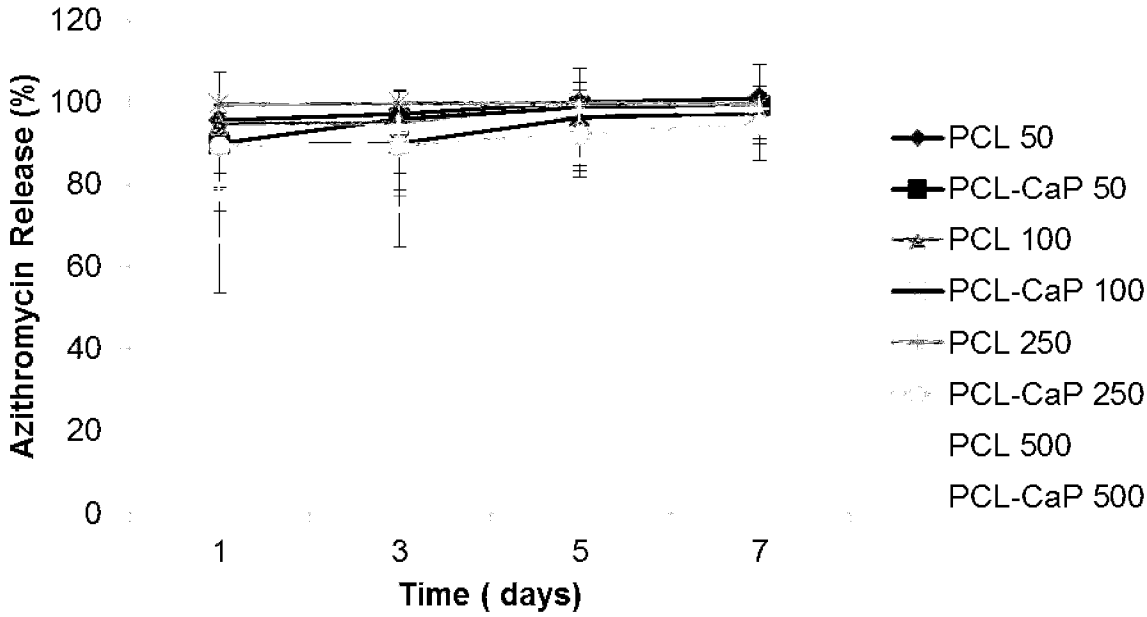
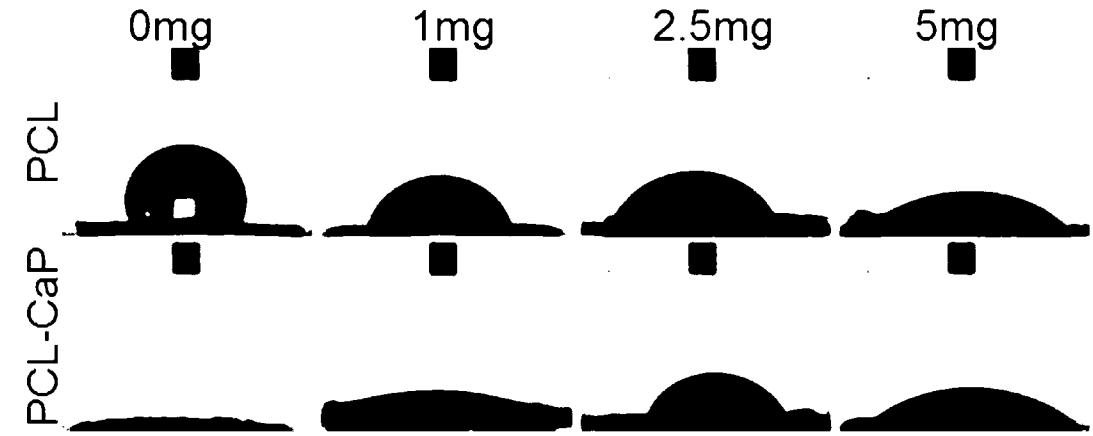
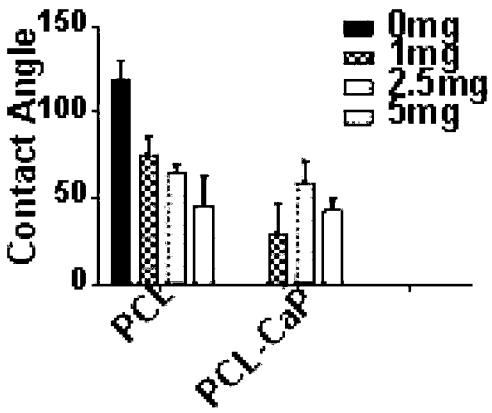


FIGURE 23

(A)



(B)



(C)

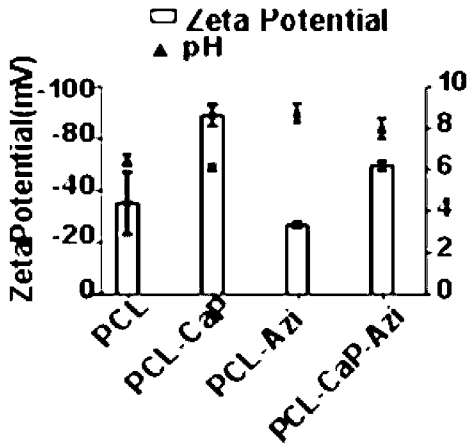
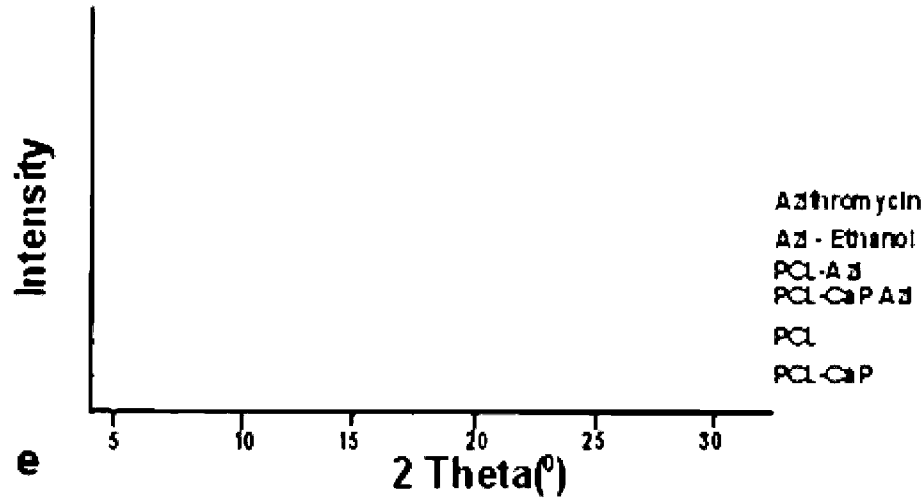


FIGURE 23 CONT'D

(D)



e

(E)

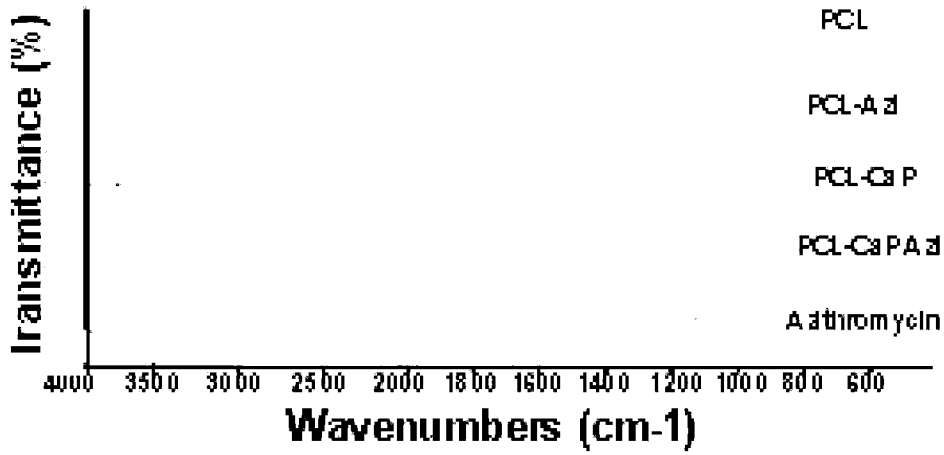


FIGURE 24

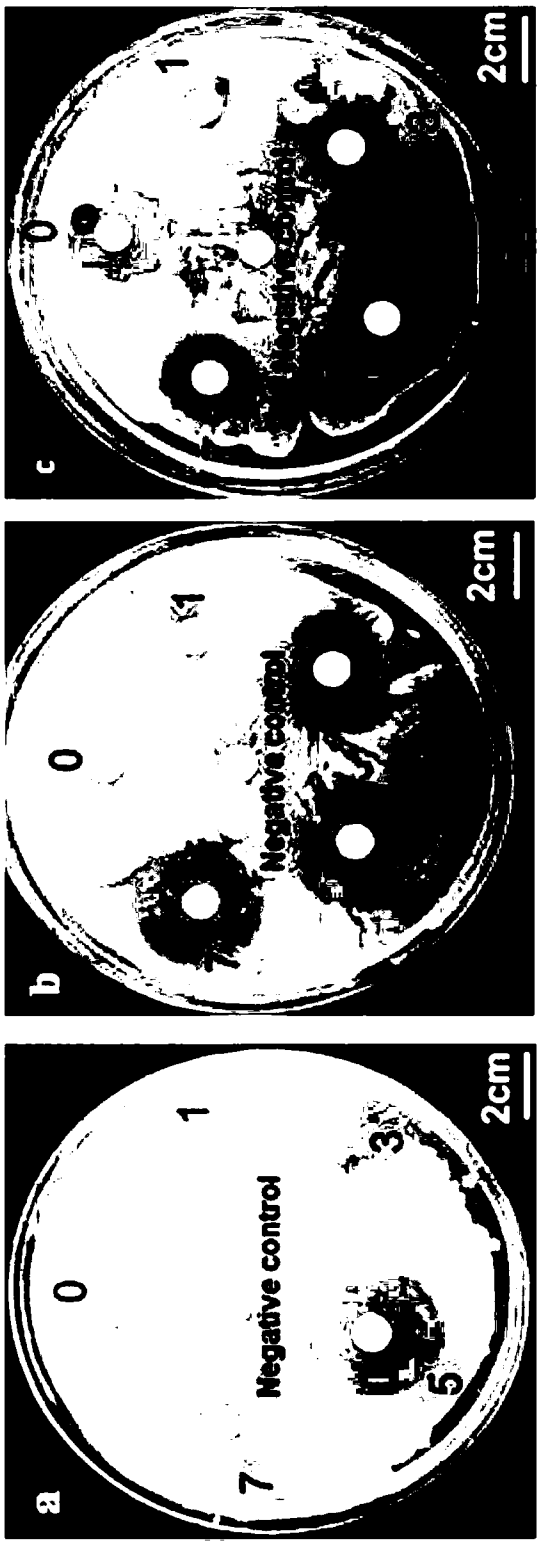

a
Negative control
0
1
3
5
7
2cm
b
Negative Control
0
1
2cm
c
Negative Control
0
1
2cm

FIGURE 25

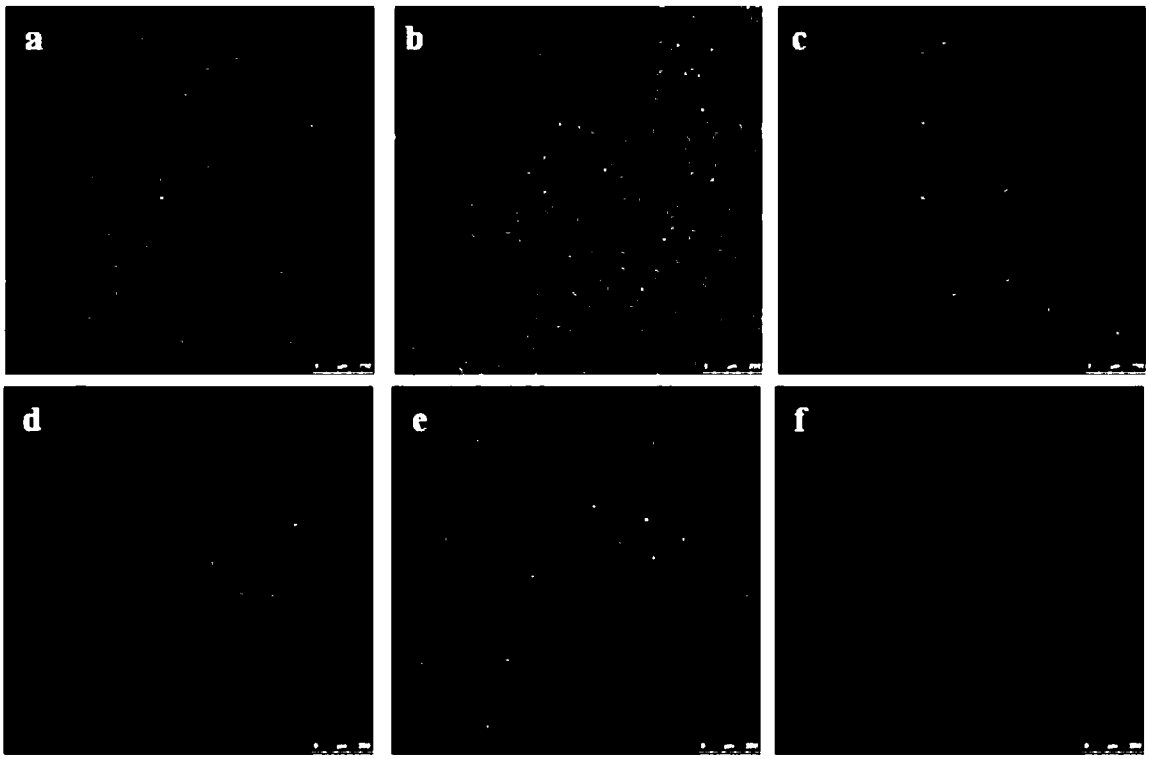
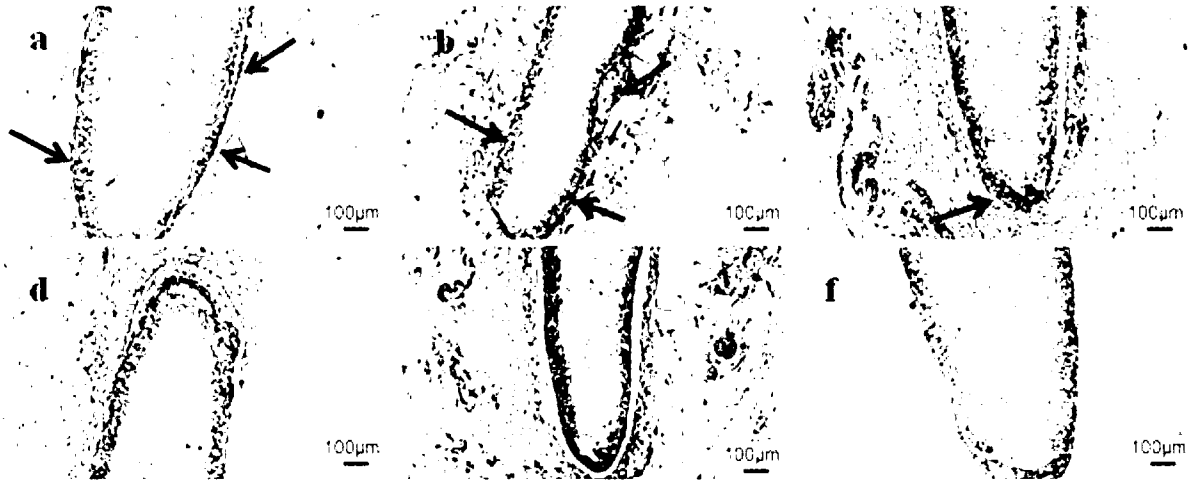


FIGURE 26


a
b
d
e
f
100µm

(g)

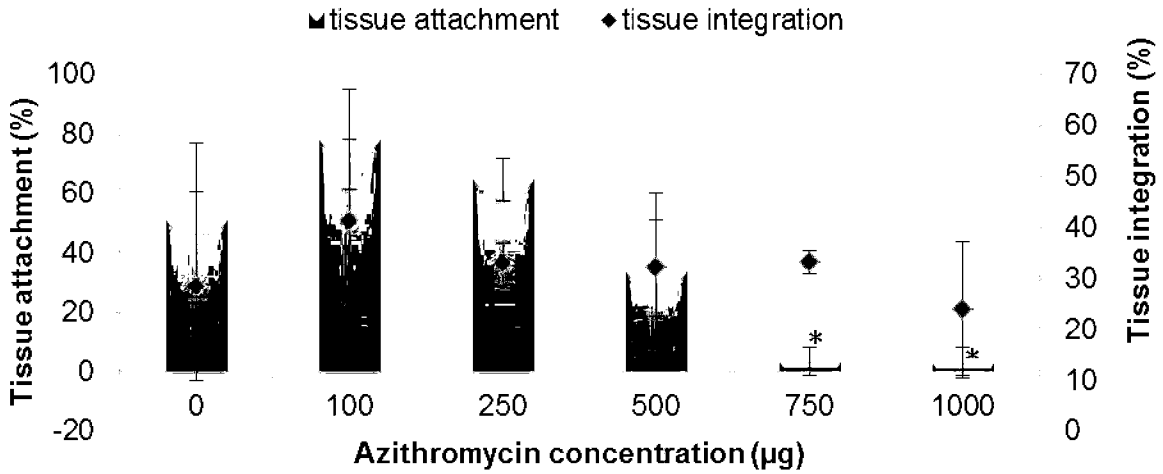
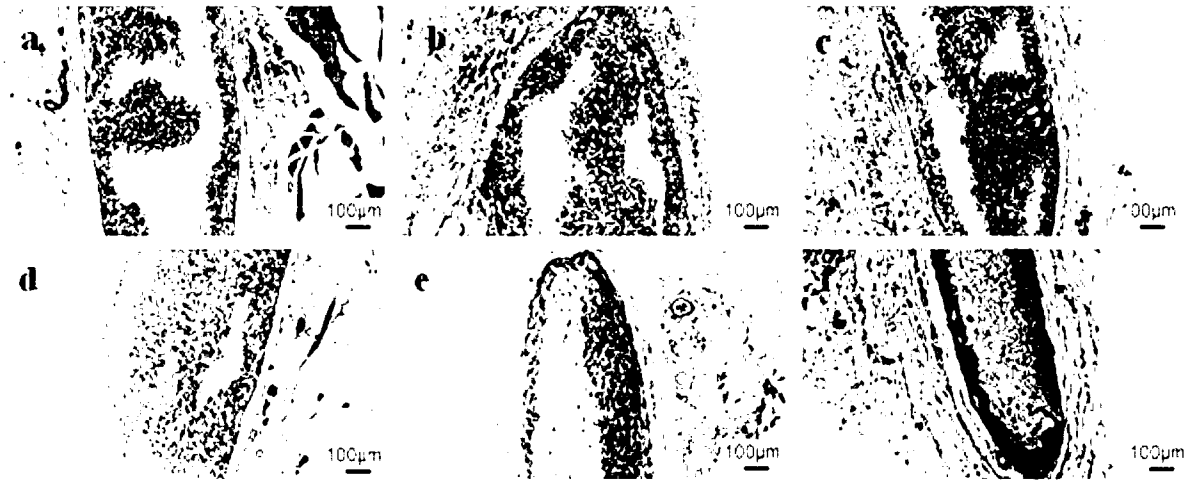

tissue attachment
tissue integration
Tissue attachment (%)
Tissue integration (%)
100
80
60
40
20
0
-20
70
60
50
40
30
20
10
0
0
100
250
500
750
1000
Azithromycin concentration (µg)

FIGURE 27


a
b
c
d
e
100µm
100µm
100µm
100µm
100µm
100µm

(g)

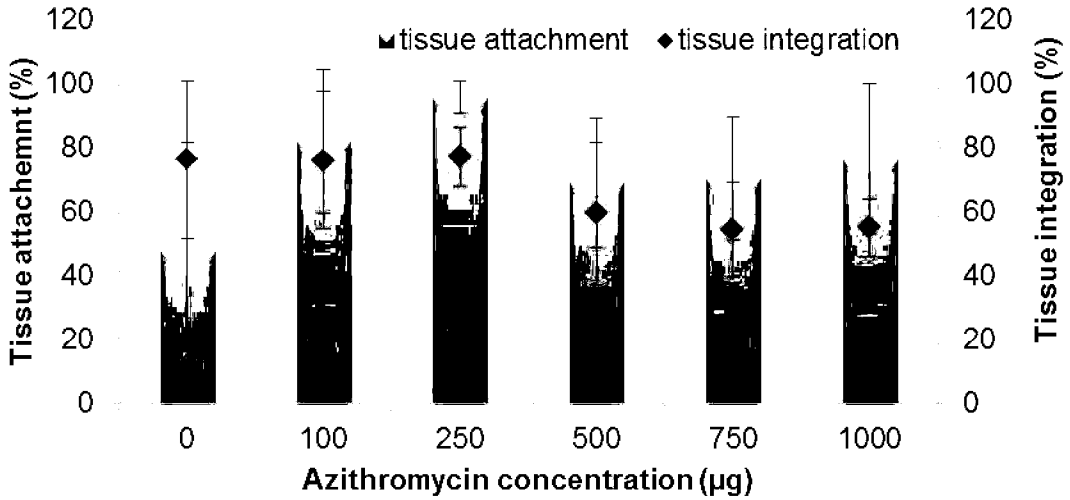
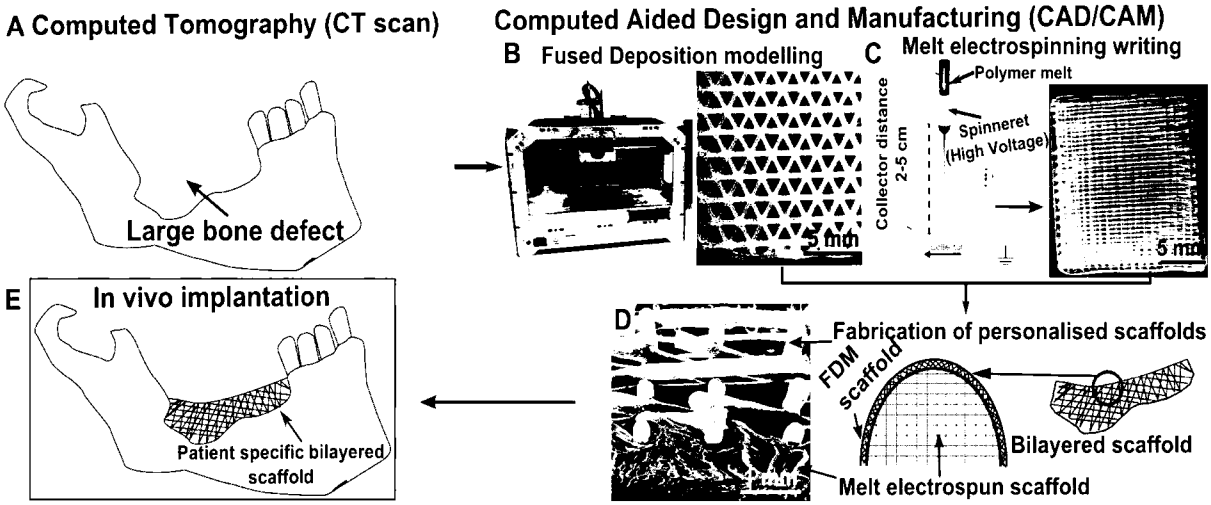

tissue attachment
tissue integration
Tissue attachemnt (%)
Tissue integration (%)
120
100
80
60
40
20
0
0
100
250
500
750
1000
Azithromycin concentration (µg)

FIGURE 28


A Computed Tomography (CT scan)
Large bone defect
Computed Aided Design and Manufacturing (CAD/CAM)
B Fused Deposition modelling
C Melt electrospinning writing
Polymer melt
Spinneret (High Voltage)
Collector distance 2-5 cm
5 mm
5 mm
E
In vivo implantation
Patient specific bilayered scaffold
D
Fabrication of personalised scaffolds
FDM scaffold
Bilayered scaffold
Melt electrospun scaffold

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2015/000592

A. CLASSIFICATION OF SUBJECT MATTER

A61L 27/38 (2006.01) ***A61L 27/44 (2006.01)*** ***A61L 27/56 (2006.01)*** ***A61C 8/00 (2006.01)*** ***A61C 13/003 (2006.01)*** ***A61F 2/28 (2006.01)***

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC & CPC Marks: A61C 8/0006, A61F 2/2803, A61L 27/3633, A61L 27/3821, A61L 27/3865, A61L 27/3604, A61C 8/0016, A61F 2002/30004

CAPLUS, MEDLINE, BIOSIS, EPODOC, WPIAP, TXTE Cluster, Google Scholar & Keywords: extracellular matrix, decellularised, coated, scaffold, osteoblast, periodontal ligament, graft, tissue regeneration, dental implant, biphasic, polymer, porous, three dimensional and like terms.

CAPLUS, MEDLINE: Applicant/Inventor name search.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 2015	30 October 2015

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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2015/000592**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	RAVINDRAN, S. et al., 'Odontogenic Induction of Dental Stem Cells by Extracellular Matrix-Inspired Three-Dimensional Scaffold', TISSUE ENGINEERING: Part A, 2014, Volume 20, Numbers 1 and 2, pages 92–102 [published 21 August 2013] Abstract, Figs. 1–3, Table 1, Introduction, Materials and Methods, pages 93, 96–97, 99 Abstract, Figs. 1–3, Table 1, Introduction, Materials and Methods, pages 93, 96–97, 99	1, 22, 24, 26–29, 56, 58, 62, 66–68, 72 1–73
Y	VAQUETTE, C. et al., 'A biphasic scaffold design combined with cell sheet technology for simultaneous regeneration of alveolar bone/periodontal ligament complex', Biomaterials, 2012, Vol. 33, pages 5560–5573 Abstract, sections 2, 3.1 & 3.2.2, Figs. 1 & 4, page 5569 right column	1–73
Y	COSTA, P. F. et al., 'Advanced tissue engineering scaffold design for regeneration of the complex hierarchical periodontal structure', J. Clin. Periodontol. 2014, Vol. 41, pages 283–294 [published 5 February 2014] Abstract, Materials and Methods, page 287 third column, Figs. 1 & 3	1–73
Y	US 2010/0292791 A1 (LU et al.) 18 November 2010 Abstract, para. [0214], [0224], [0234], [0247]–[0248], [0259], [0265], [0291], [0293], [0330], [0364], [0391], [0441], [0483]–[0507], [0514], Example 2, Tables 1, 7 & 8	1–73
Y	RAVINDRAN, S. et al., 'Biomimetic Extracellular Matrix-Incorporated Scaffold Induces Osteogenic Gene Expression in Human Marrow Stromal Cells', TISSUE ENGINEERING: Part A, 2012, Volume 18, Number 3, pages 295–309 Abstract, Introduction, Conclusion, page 295 paragraph 1, page 296 right column, pages 305–307, Fig. 1, Table 1	1–73
A	IVANOVSKI, S. et al., 'Multiphasic Scaffolds for Periodontal Tissue Engineering', J. Dent. Res., 2014, Vol. 93, No. 12, pages 1212–1221 [published 19 August 2014] Whole document	
P,A	FARAG, A. et al., 'Decellularized Periodontal Ligament Cell Sheets with Recellularization Potential', J. Dent. Res., 2014, Vol. 93, No. 12, pages 1313–1319 [published 30 September 2014] Whole document	

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2015/000592**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 2010/0292791 A1	18 November 2010	US 2010292791 A1	18 Nov 2010
		US 8753391 B2	17 Jun 2014
		EP 2155112 A1	24 Feb 2010
		EP 2187837 A1	26 May 2010
		US 2011066242 A1	17 Mar 2011
		US 8864843 B2	21 Oct 2014
		US 2010047309 A1	25 Feb 2010
		US 2015100121 A1	09 Apr 2015
		WO 2008070186 A2	12 Jun 2008
		WO 2008100534 A2	21 Aug 2008
		WO 2008154030 A2	18 Dec 2008
		WO 2008154035 A1	18 Dec 2008
		WO 2009038808 A1	26 Mar 2009

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.