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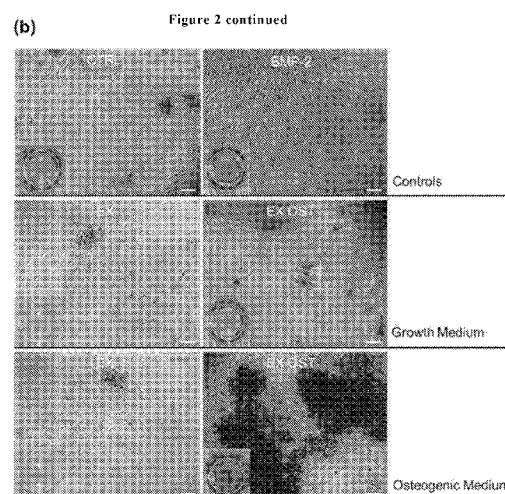
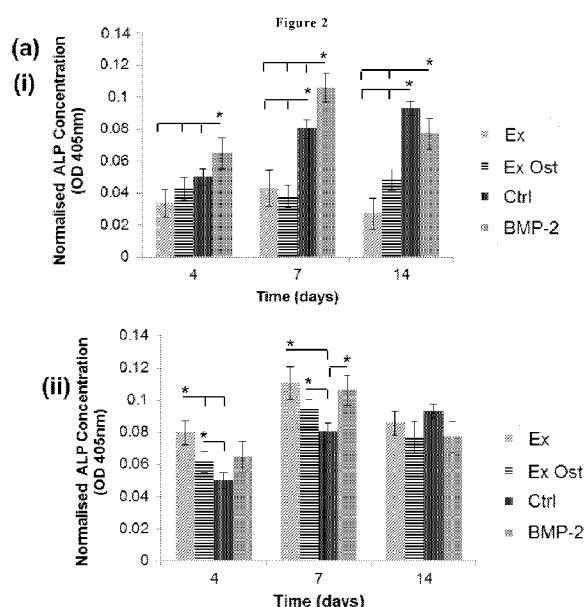
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(54) Title: COMPOSITION AND METHOD FOR BONE PRODUCTION



(57) Abstract: The present invention relates to extracellular vesicles derived from osteoblasts or osteoblast-like cells, and in particular to their use in promoting bone growth and repair.



COMPOSITION AND METHOD FOR BONE PRODUCTION

The present invention relates to methods and compositions for use in bone production including bone formation, repair or regeneration.

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Bone fractures represent a growing worldwide medical and socioeconomic burden, with 8.9 million reported annually solely as a result of osteoporosis. Despite the natural regenerative capacity of bone, there are instances where healing is impaired and clinical intervention becomes essential. Examples include when the quantity of bone required is simply beyond the body's natural regenerative capacity, such as in the skeletal reconstruction of large defects resulting from trauma or invasive surgeries (e.g. osteosarcoma excision), delayed or non-unions, or when the natural regenerative capacity is impaired due to osteoporosis or avascular necrosis. Standard clinical approaches currently used to stimulate or augment bone regeneration include distraction osteogenesis and bone transport, autologous or allogeneic bone grafts, or the application of bone graft substitutes containing hyper-concentrated growth factors such as bone morphogenetic proteins. Although each of these methods is routinely used in clinical practice with positive results observed, each suffers from limitations. For instance, autologous grafts are limited by the availability of donor tissue. Allogenic grafts present a potential source of infection and are typically stripped of many osteoinductive biological factors. Materials-based templates often result in the formation of bone that is difficult to remodel and subsequently prone to secondary fracture. Osteoinductive growth factors such as bone morphogenetic proteins (BMPs) have been the subject of recent controversy, with reports of serious negative outcomes and costly litigation. Therefore, although current interventions offer a valuable means to facilitate bone repair, none of these clinical interventions can be considered optimal and potent methods of inducing osteogenesis that are able to generate large volumes of biologically safe and mechanically resilient bone without associated patient morbidity are required.

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Modern tissue engineering approaches have been the subject of substantial research over the past two decades, with recent advances in therapies that combine osteoconductive materials with cells to provide novel ways of promoting the synthesis of new bone tissue. Cell-based approaches are appealing since they attempt to recapitulate and exploit the body's natural biological capacity for regeneration, and

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there have been a vast number of significant advances made using tissue engineering approaches. However, it has become increasingly clear that the considerable clinical value offered by cell-based approaches will be difficult to translate into clinical practice, with clinical and commercial progression frequently hindered by significant and sometimes insurmountable hurdles associated with ethics, government regulation, and high associated costs. With this in mind, there is great potential in developing a biological therapy that retains many of the benefits of a cell-based approach but without necessarily incorporating a cellular element. In the present invention, this approach is shown to be entirely possible if osteoblasts, or osteoblast-like cells, are instructed to produce extracellular vesicles comprising a heterogeneous cargo of biological factors, which can subsequently be isolated and therapeutically administered.

The importance of extracellular vesicles in cell-cell and cell-organ communication is becoming increasingly apparent, with these small heterogeneous vehicles delivering a large cargo of proteins, DNA, mRNAs, miRNAs and other important biological molecules between cells. Nowhere is the critical developmental role of extracellular vesicles more apparent than in the skeletal system, where extracellular vesicles have historically been associated with sites of early mineral formation in bone and cartilage. However, the application of extracellular vesicles as a vehicle for the delivery of bioactive factors that can be utilised for regenerative medicine is only just becoming apparent (Qin et al, 2016, *Int. J. Mol. Sci.* **17** 712). The proposition of applying extracellular vesicles for regenerative medicine has considerable benefits over traditional cell-based approaches. For instance, extracellular vesicles are subject to considerably fewer safety considerations, there is no risk of phenotypic conversion or teratogenicity upon implantation, and there are thought to be no immunological issues due to the fact that induced pluripotent stem cell-derived exosomes have previously been shown to lack MHC class I and II proteins. Given the potential benefits of extracellular vesicles, it is surprising that only a limited number of studies have sought to utilise these valuable factors for regenerative medicine applications.

An aim of the present invention is to provide extracellular vesicles with improved properties, and to provide an improved protocol/method for the administration of extracellular vesicles which results in improved bone production.

Accordingly, in a first aspect of the invention there is provided extracellular vesicles derived from osteoblasts or osteoblast-like cells comprising elevated levels of one or more of an annexin protein, osteopontin and collagen type-VI. Preferably the level of both osteopontin and collagen type-VI in the extracellular vesicles are elevated compared to levels observed in extracellular vesicles derived from non-mineralising osteoblasts or osteoblast-like cells. Preferably the level of both an annexin protein and collagen type-VI in the extracellular vesicles are elevated compared to levels observed in extracellular vesicles derived from non-mineralising osteoblasts or osteoblast-like cells.

Preferably the extracellular vesicles are derived from osteoblasts or osteoblast-like cells cultured in a mineralising environment, for example in the presence of an osteoconductive donor substrate.

The level of osteopontin observed in extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a mineralising environment may be at least 2 fold, at least 3 fold, at least 4 fold or more than that observed in extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a non-mineralising environment.

The level of collagen type-VI observed in extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a mineralising environment may be at least 2 fold, at least 3 fold, at least 4 fold or more than that observed in extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a non-mineralising environment.

In an embodiment, extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a mineralising environment have a level of osteopontin at least 4 fold more than that observed in extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a non-mineralising environment, and a level of collagen type-VI at least 3 fold more than that observed in extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a non-mineralising environment.

The annexin protein may be Annexin 2, Annexin 1 or Annexin 6. The level of one or more annexins observed in extracellular vesicles derived from osteoblasts or

osteoblast-like cells cultured in a mineralising environment may be at least 2 fold, at least 3 fold, at least 4 fold or more than that observed in extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a non-mineralising environment. The level of Annexin 2 observed in extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a mineralising environment may be at least 2 fold, at least 3 fold, at least 4 fold or more than that observed in extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a non-mineralising environment.

Extracellular vesicles derived from osteoblasts or osteoblast-like cells cultured in a mineralising environment may have Annexin 1 and/or Annexin 6 which are otherwise absent from extracellular vesicles derived from osteoblasts or osteoblast-like cells.

Osteoblast-like cells include odontoblasts (located within dentine), ameloblasts (located within enamel), cementoblasts (located within cementum) and skeletal muscle cells.

In a further aspect the invention provides a composition comprising extracellular vesicles according to the first aspect of the invention.

In another aspect of the invention, there is provided a pharmaceutical composition comprising extracellular vesicles derived from osteoblasts or osteoblast-like cells and a pharmaceutically acceptable carrier. Preferably the composition is intended for administration with an osteoconductive donor substrate. The extracellular vesicles may be as described with reference to the first aspect of the invention.

In another aspect of the invention, there is provided a composition comprising extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate. Preferably the composition is a pharmaceutical composition and further comprises a pharmaceutically acceptable carrier. The extracellular vesicles may be as described with reference to the first aspect of the invention.

In another aspect of the invention, there is provided a method of promoting bone formation/regeneration in a subject at a site in need thereof, the method comprising the steps of:

- (i) obtaining extracellular vesicles obtained from osteoblasts or osteoblast-like cells;
- (ii) administering the extracellular vesicles to a site in a subject where the promotion of bone formation/regeneration is required; wherein the extracellular vesicles are administered with one or more osteoconductive donor substrates.

Preferably the extracellular vesicles obtained in step (i) were obtained from osteoblasts cultured in the presence of one or more osteoconductive donor substrate.

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The extracellular vesicles and osteoconductive donor substrate may be administered sequentially, simultaneously or separately. The extracellular vesicles may be as described with reference to the first aspect of the invention.

- 15 In a further aspect of the invention, there is provided a method of promoting bone formation/regeneration *in vitro*, the method comprising the steps of:

- (i) obtaining extracellular vesicles obtained from osteoblasts or osteoblast-like cells;
- (ii) administering the extracellular vesicles to cells *in vitro*; wherein the extracellular vesicles are administered with one or more osteoconductive donor substrate.

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Preferably the extracellular vesicles obtained in step (i) were obtained from osteoblasts or osteoblast-like cells cultured in the presence of one or more osteoconductive donor substrate.

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The extracellular vesicles and osteoconductive donor substrate may be administered sequentially, simultaneously or separately. The extracellular vesicles may be as described with reference to the first aspect of the invention.

- 30 The cells may be autologous or heterologous stem cells, osteoblasts or osteoblast-like cells, preferably the stem cells, osteoblasts or osteoblast-like cells are autologous to the subject to whom they will be administered.

- 35 This method may be used to induce mineralisation in the cells, that is, the early stages of bone formation, prior to administration of the cells to a subject. In an embodiment

the cells, such as stem cells, osteoblasts or osteoblast-like cells may be seeded on a scaffold comprising an osteoconductive donor substrate, thereby allowing the production of a scaffold primed for bone regeneration before it is administered.

- 5 The *in vitro* method described may be used with any standard tissue engineering method/protocol.

In a further aspect the invention provides a method of treating a bone condition in a subject in need of bone regeneration, or of promoting bone formation in a subject
10 where needed, comprising locally administering to the subject a therapeutically effective amount of extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate. The extracellular vesicles and osteoconductive donor substrate may be administered sequentially, simultaneously or separately. The extracellular vesicles and osteoconductive donor substrate may be in
15 the same composition and administered simultaneously, or they may be in separate compositions and may be administered simultaneously, sequentially or separately. The extracellular vesicles may be as described with reference to the first aspect of the invention.

20 In another aspect the invention provides use of extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate in the manufacture of a medicament for use in the treatment of a bone condition or in promoting bone growth/regeneration. The extracellular vesicles may be as described with reference to the first aspect of the invention.

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In a further aspect the invention provides extracellular vesicles derived from osteoblasts or osteoblast-like cells for use in the treatment of a bone condition or in promoting bone growth/regeneration, wherein the extracellular vesicles derived from osteoblasts or osteoblast-like cells are intended to be administered with an
30 osteoconductive donor substrate. The extracellular vesicles and osteoconductive donor substrate may be intended to be administered sequentially, simultaneously or separately. The extracellular vesicles may be as described with reference to the first, or any, aspect of the invention.

In a further aspect the invention provides a composition comprising extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate for use in the treatment of a bone condition or in promoting bone growth/regeneration. The extracellular vesicles may be as described with reference to
5 the first, or any, aspect of the invention.

In a yet further aspect the invention provides a kit comprising extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate and instructions for administration to a subject to treat a bone condition
10 and/or to aid in bone growth/regeneration. The extracellular vesicles and osteoconductive donor substrate may be provided in the same composition for administration together. Alternatively the extracellular vesicles and osteoconductive donor substrate may be provided in separate compositions that are mixed prior to administration. Alternatively the extracellular vesicles and osteoconductive donor
15 substrate may be provided in separate compositions and may be administered separately. If administered separately the extracellular vesicles and osteoconductive donor substrate may be administered within 10 minutes of each other, for example if both are administered in a fluid form. Alternatively, if the osteoconductive donor substrate is administered as a solid scaffold, perhaps during an invasive procedure, the
20 extracellular vesicles may be administered several hours or even days later, for example by injection into the scaffold. The extracellular vesicles and osteoconductive donor substrate may be intended for local administration. The kit may further comprise a biocomposite, such as a scaffold or cement. The biocomposite may be mixed with the extracellular vesicles and an osteoconductive donor substrate prior to
25 administration. Alternatively, the biocomposite may comprise the osteoconductive donor substrate, in which case it may be mixed with the extracellular vesicles prior to administration or it may be administered and the extracellular vesicles may be administered separately.

30 The extracellular vesicles may be as described with reference to the first aspect of the invention.

In another aspect the invention provides an implantable device, such as a scaffold, cement or fixation device, which carries or contains extracellular vesicles and/or an
35 osteoconductive donor substrate. The implantable device may include orthopaedic

and spinal implants, including screws, plates, rods, pins, hooks, anchors, intramedullary devices, pedicle screws, pedicle hooks, spinal fusion cages, prostheses, porous metal implants such as trabecular metal implants and the like. The extracellular vesicles and/or osteoconductive donor substrate may be coated on the surface of, or applied as a surface modification to, an implantable device by conventional means. Alternatively, the implant may be formulated and fabricated such that the extracellular vesicles and/or osteoconductive donor substrate is incorporated into the implant. Means by which this can be accomplished are well known to those of ordinary skill in the art. The extracellular vesicles may be as described with reference to the first aspect of the invention.

In an aspect of the invention the extracellular vesicles may be exosomes and/or macrovesicles. The extracellular vesicles may be exosomes.

The extracellular vesicles may have a diameter up to about 300nm, up to about 200nm, up to about 150nm, up to about 125nm, or up to about 100nm. The extracellular vesicles may have a diameter of between about 30nm and about 200nm.

In embodiments where the extracellular vesicles are exosomes, the exosomes may have a diameter of up to about 150nm, up to about 125nm, up to about 100nm. The exosomes may have a diameter of between about 10nm and about 150nm, between about 20nm and about 150nm, between about 30nm and about 150nm, between about 40nm and about 125nm, between about 50nm and about 100nm.

The osteoconductive donor substrate may be a mineral donating substrate. The donating substrate may comprise calcium, phosphorus, magnesium, sodium, chloride, pyrophosphate, potassium, fluoride, strontium, zinc, chromium, cobalt, copper, manganese, silicon, carbonate or bicarbonate. Preferably the mineral is calcium or phosphorus. The mineral donating substrate may be calcium phosphate, hydroxyapatite, calcium deficient hydroxyapatite, tetracalcium phosphate, alpha-tricalcium phosphate, beta-tricalcium phosphate, octacalcium phosphate, calcium pyrophosphate, dicalcium phosphate dihydrate, dicalcium phosphate anhydrous, beta-glycerophosphate, calcium beta-glycerophosphate or sodium beta-glycerophosphate.

In an embodiment the extracellular vesicles are administered to a subject in the presence of one or more of beta-glycerophosphate, calcium beta-glycerophosphate or sodium beta-glycerophosphate. The beta-glycerophosphate, calcium beta-glycerophosphate or sodium beta-glycerophosphate may be provided at a concentration of between about 0.1mM and about 100mM beta-glycerophosphate, preferably between about 1mM and about 100mM, preferably between about 5mM and about 50mM, more preferably about 10mM beta-glycerophosphate. The extracellular vesicles may also be administered with ascorbic acid. The ascorbic acid may be included at a concentration of between about 1µg/ml and about 200µg/ml, preferably about 30µg/ml and about 100µg/ml, preferably about 50µg/ml.

Preferably in a composition or method of the invention extracellular vesicles from sources other than osteoblasts or osteoblast-like cells are removed. This can be achieved by centrifuging solutions to remove unwanted extracellular vesicles prior to the addition of the extracellular vesicles derived from osteoblasts or osteoblast-like cells.

The osteoblast cells or osteoblast-like cells from which the extracellular vesicles are derived may be mammalian osteoblast or osteoblast-like cells. The osteoblast or osteoblast-like cells from which the extracellular vesicles are derived may be human osteoblast or osteoblast-like cells. The osteoblast or osteoblast-like cells from which the extracellular vesicles are derived may be mouse osteoblast or osteoblast-like cells.

The extracellular vesicles may be obtained from osteoblasts or osteoblast-like cells by stimulating the osteoblasts or osteoblast-like cells to produce vesicles by culturing the osteoblasts or osteoblast-like cells in a growth medium containing an osteoconductive donor substrate – sometimes referred to as a mineralising environment. Preferably the extracellular vesicles are produced in the absence of dexamethasone. The extracellular vesicles may be isolated from the growth medium using various techniques known in the art, including filtration, centrifugation, ion-chromatography or concentration.

The extracellular vesicles of the invention may be obtained from osteoblasts or osteoblast-like cells cultured in a growth medium containing between 0.1mM and about 100mM beta-glycerophosphate, preferably between about 1mM and about

100mM, preferably between about 5mM and about 50mM, more preferably about 10mM beta-glycerophosphate. The medium may also comprise ascorbic acid. The ascorbic acid may be included at a concentration of between about 1µg/ml and about 200µg/ml, preferably about 30µg/ml and about 100µg/ml, preferably about 50µg/ml.

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Accordingly, the invention may provide a method of producing extracellular vesicles. The method may further include the step of recovering and isolating the extracellular vesicles produced. The extracellular vesicles may be recovered or isolated by using differential centrifugation, ultracentrifugation, precipitation, chromatography or sequential filtration. Extracellular vesicles produced by this method may be useful for hard tissue regeneration, for example bone regeneration. By controlling the environment in which the vesicles are produced the pro-osteogenic effects of the vesicles produced can be improved.

15 The isolated extracellular vesicles may include one or more types of extracellular vesicles. For example, the isolated extracellular vesicles may comprise one, two, three four, five, six, seven, eight, nine, ten or more types of exosomes. The types of extracellular vesicles may be characterised by their compositions, for example, by the nucleic acids and/or proteins contained in the extracellular vesicles.

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An osteoconductive donor substrate may be administered simultaneously with the extracellular vesicles, in the same composition or in a separate composition. Alternatively the osteoconductive donor substrate and extracellular vesicles may be administered sequentially, that is the osteoconductive donor substrate may be administered just before or just after administration of the extracellular vesicles. The osteoconductive donor substrate may be administered 10 minutes before or after administration of the extracellular vesicles. In another embodiment the osteoconductive donor substrate may be administered several hours, or even days, before administration of the extracellular vesicles. For example, the osteoconductive donor substrate may form part of a scaffold/cement which may be administered minutes, hours or days before the administration of the extracellular vesicles. The scaffold/cement comprising the osteoconductive donor substrate may be administered during an invasive procedure to a site in need of bone formation/regeneration, the extracellular vesicles may then be administered either during the invasive procedure

or later, for example by injection into the scaffold/cement or to the site where bone formation is needed.

5 The extracellular vesicles, and optionally an osteoconductive donor substrate, may be administered in combination with one or more of autograft bone, allograft bone, autologous stem cells, allogenic stem cells, autologous growth factors, allogenic growth factors, chemical stimulation methods, electrical stimulation methods, low-intensity pulse ultrasound methods, internal fixation methods, external fixation methods, a bone scaffold and an orthopaedic biocomposite. In an embodiment the
10 extracellular vesicles may be administered with a biocomposite or scaffold which comprises an osteoconductive donor substrate, for example a mineral composite such as a calcium phosphate scaffold or cement. In an embodiment the extracellular vesicles and/or the osteoconductive donor substrate may be admixed with a biocomposite, such as a cement or a scaffold, prior to administration to a subject.

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The extracellular vesicles and/or osteoconductive donor substrate may also be intended to be administered with one or more further agents, wherein the further agents may be selected from a cytotoxic agent, a cytokine, a growth inhibitory agent, an further bone growth stimulator, a peptide growth factor, an anti-inflammatory
20 agent, a pro-inflammatory agent, an inhibitor of apoptosis, an MMP inhibitor and a bone catabolic antagonist such as a bisphosphonate, a osteoprotegerin or a statin.

In some embodiments of the invention, the extracellular vesicles may be loaded with externally added therapeutic agents. Suitable therapeutic agents include pro-
25 angiogenic agents such as VEGF, angiopoietin and PDGF; trace elements such as Cr, Si, Zn, Cu, S and bioactive glasses; antibacterial agents such as antibiotics and silver; bone growth stimulators such as BMPs, TGF-beta, PDGF; and microRNAs such as miR-218, miR-96, miR-199b, miR-133, miR-135, miR-26, and miR-29.

30 In alternative embodiments the extracellular vesicles are not loaded with an external therapeutic agent.

The extracellular vesicles according to the invention may act as independent sites of mineralisation (independent of cells). The extracellular vesicles may allow the uptake

and processing of one or more of calcium, phosphate and magnesium ions required for mineralisation.

The subject may be a mammalian animal, in particular a human. The subject may
5 alternatively be a horse, dog, cat or other domestic animal.

The present invention may lead to newly formed bone that has improved bone quality, quantity and density, in particular in comparison to compromised bone which may otherwise be produced at a site of injury. The data presented herein uses X-ray
10 fluorescence elemental mapping to show for the first time that there is enhanced calcium deposition within stem cell cultures treated with extracellular vesicles derived from mineralising osteoblasts and administered with an osteoconductive donor substrate. Furthermore, calcium and phosphorus ions are shown to co-localise when
15 extracellular vesicles derived from mineralising osteoblasts are administered to stem cell cultures with an osteoconductive donor. Co-localisation was absent within stem cell cultures treated with BMP-2 (current clinical gold-standard) or extracellular vesicles administered in the absence of an osteoconductive donor substrate. These
20 results indicate that the mineral formed in the presence extracellular vesicles derived from mineralising osteoblasts and administered with an osteoconductive donor substrate is likely to be at a more advanced stage than that formed in the other examples. Mineral is thought to transition through a number of intermediate and less
25 stable forms on the pathway to end-stage hydroxyapatite. The data presented herein indicates that mineral formed in the presence of extracellular vesicles and an osteoconductive substrate is further advanced along this transition than the other examples.

The method or composition of the invention may be used at, or adjacent to, a site in need of the promotion of bone formation/regeneration. This site may be any of a number of areas including bone that is injured, damaged, fractured, eroded, brittle, or
30 defective in some other way that would benefit from the promotion of bone formation/regeneration at that site. The site may be a site of injury, such as a fracture of a bone. The site may be a site of surgical intervention, such as insertion of an implant into a bone. The site may a site of disease, pathology, condition or disorder. The site may be a bone affected by osteoporosis.

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The method or composition of the invention may be used to promote bone formation/regeneration in a subject afflicted with a bone condition. The bone condition may be selected from bone fracture; osseous defects: delayed unions; non-unions; bone trauma; arthrodesis including spinal arthrodesis, extremity arthrodesis and the like; a bone deficit condition associated with post-traumatic bone surgery, post-prosthetic joint surgery, post-plastic bone surgery, post-dental surgery, bone chemotherapy treatment, congenital bone defect, post-traumatic bone loss, post-surgical bone loss, post infection bone loss, allograft incorporation or bone radiotherapy treatment; and osteoporosis.

The method or composition of the invention may be used to promote fusion of two bones or bone fragments. The method or composition of the invention may be used to promote spinal fusion, for example to treat lumbar degenerative disc disease. The composition may be administered via a spinal cage which may be used to provide load bearing support to the vertebrae.

The method or composition of the invention may be used in dental applications, for example to promote bone growth around a dental implant. In particular, the method or composition of the invention may be used to promote maxillofacial repair/regeneration and/or to enhance dentine production for the treatment of caries. The method or composition of the invention may also be used to promote osteointegration of metallic or polymeric dental implants, including dental implants for crowns (abutment and abutment screws) and dental bridges.

The dosage of the extracellular vesicles and an osteoconductive donor substrate administered will depend on the intended application. The determination of the appropriate dosage and route of administration is well within the skill of the ordinary physician.

The extracellular vesicles and/or osteoconductive donor substrate may be administered directly to a site where needed, this may be achieved by injection into the site or adjacent to the site, alternatively the extracellular vesicles and/or osteoconductive donor substrate may be administered surgically as a paste, putty, gel or cement, in combination with a scaffold, as part of an implant, in a viscous fluid or a polymeric solution.

The extracellular vesicles and/or osteoconductive donor substrate may be administered entrapped in micro-particles, in a semipermeable matrix or in a biodegradable material such as a hydrogel.

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The extracellular vesicles and/or an osteoconductive donor substrate may be administered in a form that allows immediate release, controlled release, sustained release or extended released.

10 In a further aspect, the present invention provides a method and medium for delivering the extracellular vesicles of the invention. The extracellular vesicles of the invention may be delivered by suspending them in a liquid phase containing polymer fluid gel microparticles as described with reference to cells is WO2014/140549. In an embodiment the extracellular vesicles are not embedded within the microparticles.

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The rheological properties of the fluid gels "liquid phases" can be controlled to allow these systems to have a range of flow functionalities when applied at a desired location where they should be retained. Properties of the fluid gels can be tailored to each specific application (to be injectable, spreadable, shear-thinning, Newtonian, etc)
20 by controlling the formulation and processing parameters involved during their manufacture.

The fluid gel structure itself as well as its rheological properties can be also carefully formulated, if required by specific applications for these systems, to be transient
25 rather than stable. For example, a fluid gel structure can be designed to revert to a typical (non-sheared) gel structure as a function of time, external stimuli (temperature, ionic charge, etc.). This can allow for the formulation of a fluid gel structure carrying extracellular vesicles that is initially injectable but subsequently, e.g. after application at the point of injury, forms a structure that is firmly anchored but has been shaped to
30 occupy the available space in the body (e.g. not causing any disruption to movement) and still retains the extracellular vesicles. Similarly the actual flow behaviour of the fluid gels can be designed to be transient. This can allow for specific flow characteristics (e.g. a high yield stress, etc.) initially exhibited by the system to be either irreversibly or reversibly altered following application; for example, in the case
35 of a system exhibiting a high yield stress, this behaviour can be temporarily

eliminated by the application of shear (while it is injected) during application, but once shear is removed the yield stress functionality returns after a certain (and again controllable) "resting period".

- 5 The invention provides, an extracellular vesicles delivery medium comprising a liquid phase, wherein the liquid phase comprises (i) extracellular vesicles suspended (or entrapped) within the liquid phase and (ii) a plurality of polymer gel particles. Typically the extracellular vesicles are not entrapped within the polymer gel particles but are within the liquid phase. Extracellular vesicles within both the liquid phase and
10 the gel particles may also be provided.

A further aspect of the invention provides an extracellular vesicles delivery medium comprising an osteoconductive donor substrate, extracellular vesicles and a plurality of polymer gel particles, wherein said polymer gel particles do not encapsulate the
15 extracellular vesicles.

The liquid phase may be any suitable liquid, especially aqueous liquid, for suspending extracellular vesicles. Preferably the liquid phase comprises an osteoconductive donor substrate.

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The polymer gel may be selected from agarose, agar, carrageenan, gellan gum, gelatin, pectin, alginate and fibrin. Non-naturally occurring gels, for example, polyacrylate and polyethylene glycol, may be used. Other suitable gels include chitosan, dextran, collagen and hyaluronic acid. The particles may be substantially spherical, needle or
25 threadlike. The particles may be within substantially a single size distribution family or within several discrete size distribution features.

The polymer gel may be a hydrogel. Hydrogels typically are networks of polymer chains that are hydrophilic, and are sometimes found as gel in which water is the dispersion medium. Examples of hydrogels include gelatin, poly(alkylene oxides) such
30 as poly(ethylene oxide), poly(meth)acrylates and methyl cellulose. Most typically, the hydrogel comprises a gellan, a generally known polysaccharide gum which is produced by *Pseudomonas elodea*.

Gellan may be used at 0.5% -3% wt/vol of the formulation prior to setting, for example 1-2.5% wt/vol or 2% wt/vol.

Alginate may also be used.

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Mixtures of gellan and alginate may be used, for example at 0.5%-1% gellan to 0.25-0.75% alginate.

10 Gellan gum is based on a linear structure of repeating glucose rhamnose and glucuronic acid units. In high acyl gum, two acyl side chains of acetate and glycerate are present. Both substituents are present on the same glucose molecule and on average there is one glycerate per repeating unit and one acetate every two repeating units. In low acyl gellan gums the acyl groups are removed. High acyl products tend to form soft elastic gels while gellan gum produces firmer, less elastic gels. The gellan
15 gum may be high or low acyl or a combination thereof.

Typically the average size of the polymer gel particles is 1 to 1000 μ m, 1 μ m to 500 μ m or 10 μ m to 100 μ m, or 30 μ m to 50 μ m.

20 The extracellular vesicles delivery medium, according to the invention, may utilise in the polymer gel and/or liquid phase, one or more additional agents such as antibiotics, hormones, growth factors or anti-inflammatory compounds.

A further aspect of the invention provides an extracellular vesicles delivery medium
25 according to the invention for use in the treatment of a bone condition. The bone condition may be selected from bone fracture; osseous defects: delayed unions; non-unions; bone trauma; arthrodesis including spinal arthrodesis, extremity arthrodesis and the like; a bone deficit condition associated with post-traumatic bone surgery, post-prosthetic joint surgery, post-plastic bone surgery, post-dental surgery, bone
30 chemotherapy treatment, congenital bone defect, post-traumatic bone loss, post-surgical bone loss, post infection bone loss, allograft incorporation or bone radiotherapy treatment; and osteoporosis. The bone condition may be a dental condition as previously described.

The extracellular vesicles delivery medium of the invention may be used to promote fusion of two bones or bone fragments. The method or composition of the invention may be used to promote spinal fusion, for example to treat lumbar degenerative disc disease. The composition may be administered via a spinal cage which may be used to
5 provide load bearing support to the vertebrae.

The invention also provides methods of treatment using the extracellular vesicles delivery medium according to the invention.

10 A further aspect of the invention provides a method of producing an extracellular vesicles delivery medium comprising:

- dissolving a gelling polymer gel in a liquid phase to form a mixture existing in a liquid state;
- inducing gelation of the polymer gel liquid mixture under application of
15 shear to form a mixture comprising a plurality of polymer gel fluid gel particulates within a liquid phase; and
- adding extracellular vesicles to form a suspension of the extracellular vesicles within the liquid phase.

20 The invention also provides a method of producing an extracellular vesicles suspension medium comprising:

- (i) heating a polymer gel in a liquid phase to above the melting temperature of the polymer gel to form a heated mixture; and
- (ii) cooling the heated mixture under shearing to form a mixture comprising a
25 plurality of polymer particulates within the liquid phase.

Extracellular vesicles may be provided in one or both of the liquid phase and particulates.

30 The mixture comprising the plurality of polymer gel microparticles may be sterilised by irradiation, typically prior to addition of the extracellular vesicles. Irradiation may utilise, for example, ultraviolet, x-ray or gamma ray radiation to sterilise the medium, for example, to remove unwanted bacterial contamination.

35 Typically, the shearing is induced by passing the heated mixture through a pin stirrer

as it is cooled. For example, the media may be passed through a water bar to cool the extracellular vesicles that are not encapsulated within a polymer gel matrix, but are suspended within the liquid phase of a fluid gel system and surrounded by individual fluid gel microparticles, fluid gels with advantageous properties can be produced. This allows, for example, the extracellular vesicles to be mobile within a flowable, spreadable or injectable solution depending on the processing characteristics of the fluid gel component. Indeed, the properties of the fluid gels can be tailored for each specific application by controlling the formulation and processing parameters involved during the manufacture. This means that there is the ability to specifically design the functionality of the extracellular vesicles carrying system for use in different scenarios and applications.

The skilled person will understand that optional features of one embodiment or aspect of the invention may be applicable, where appropriate, to other embodiments or aspects of the invention. For example, embodiments or aspects discussed in the context of methods, uses and/or compositions of the invention may be employed with respect to any other method, use and/or composition described herein.

There now follows by way of example only a detailed description of the present invention with reference to the accompanying drawings, in which;

Figure 1 shows the profiling and characterisation of exosomes isolated from osteoblasts cultured in a mineralising environment. Figure 1a shows topographic profiling of exosomes using AFM under tapping mode. Figure 1b shows tunable resistive pulse sensing measurement of exosome size using Izon qNano. Figure 1c shows ImageStream detection of lipid membrane (FM 1-43FX) and transmembrane surface protein (CD9).

Figure 2 shows the osteoinductive effects of exosomes administered according to the invention. Figure 2a shows alkaline phosphatase (ALP) analysis at days 4, 7, and 14 in (i) the presence of exosomes (Ex); and (ii) the presence of exosomes and an osteoconductive donor substrate. Figure 2b shows Alizarin red calcium staining in hMSC cultures exposed to exosomes.

Figure 3 illustrates micro-XRF mapping of calcium and phosphorus in hMSC cultures treated with exosomes according to the invention and as depicted in Figure 1.

5 **Figure 4** shows upregulation of the expression of type-VI collagen (Col6a1, Col6a2 and Col6a3) and osteopontin (Spp1) in exosomes derived from mineralising osteoblasts when compared with exosomes derived from non-mineralising osteoblasts.

10 **Figure 5** shows that extracellular vesicles of the invention can act as independent sites of mineralisation. Transmission electron microscopy with accompanying energy dispersive x-ray spectroscopy (EDX) demonstrates localisation of elements critical in mineralisation to extracellular vesicles. To obtain this data a suspension of extracellular vesicles according to the invention was made in sterile calcium-free PBS. A single drop of the suspension (~5µL) was deposited onto a carbon film grid (Agar Scientific, UK) and left to air dry for a period of 1 minute. To remove excess PBS the grid was gently blotted using tissue paper through capillary action. Samples were imaged using a JEM-2100 PLUS transmission electron microscope (Joel, USA) using a voltage of 80 kV. Energy dispersive x-ray spectroscopy was performed on individual extracellular vesicles (Oxford INCA).

25 **Figure 6** – is a schematic highlighting the main stages in the manufacture and isolation of pro-osteogenic extracellular vesicles and the two alginate delivery systems developed for local delivery. Figure 6A shows alginate fluid gels notably with a cross-linked gel phase and a continuous alginate phase which is not cross-linked; Figure 6B shows alginate micro-particles produced by vibrating nozzle technology.

30 **Figure 7** – illustrates the formulation and rheological characterisation of an alginate fluid gel. Figure 7A shows a range of fluid gel parameters used for fluid gel formulations, Figures 7B and C show the influence of agitator speed on shear thinning behaviour, Figures 7D and E show the influence of agitator speed on the frequency dependent response of fluid gels, and Figure 7F shows

the influence of the calcium chloride to alginate ratio on force required to inject fluid gels

5 **Figure 8** - depicts a homogeneous distribution of extracellular vesicles loaded within a) alginate microparticles (left), and b) alginate fluid gel (right). Extracellular vesicles were labelled with FM1-43FX lipid dye to allow for visualisation using a confocal microscopy. 3D images were produced by stacking 2D images.

10 **Figure 9** – shows the co-localisation of SYTO ENA select dye (staining extracellular vesicle RNA content) and FM1-43FX lipid dye (staining phospholipid EV membrane).

15 **Figure 10** – shows the release of extracellular vesicles from alginate microparticles and fluid gel systems A) NTA profile of extracellular vesicles prior to incorporation into delivery systems, B) Cumulative release of extracellular vesicles from micro particles and fluid gel quantified using NTA, C) Size profiles of extracellular vesicles released from microparticles and fluid gel up to 5 hours measured using NTA including tabulated information of mean and median sizes for each sample and time point, D) Longer term release of extracellular vesicles from fluid gel system quantified using ExoELISA – CD63.

25 MATERIALS AND METHODS

Cell Culture and Reagents

Human bone marrow-derived human mesenchymal stem cells (hMSCs) were extracted and purified from a young Caucasian male (Lonza, <http://www.lonza.com>). hMSCs were fully profiled for the expression of CD29, CD44, CD105 and CD166 and lack of CD14, CD34, CD45 expression. MC3T3-E1 murine osteoblasts were purchased from America Type Culture Collection (ATCC, <http://www.atcc.org>). Cell culture medium comprised of minimal essential medium (α -MEM) supplemented with 10% foetal bovine serum (FBS), L-glutamine (Sigma) and 1% penicillin/streptomycin (Sigma).

35 hMSCs were used between passage 4-5. Osteogenic medium was formulated through

the addition of 10mM β -glycerophosphate (Sigma) and 50 μ g/mL L-ascorbic acid (Sigma). Culture medium used for exosome isolation was depleted of any contaminating exosomes by ultra-centrifuging the FBS at 120,000g for 70 minutes prior to use.

5

Exosome Isolation and Characterisation

MC3T3-E1 osteoblasts were cultured at scale in T175 culture flasks and the medium isolated every two days for extracellular vesicle isolation. Osteoblasts were grown in either standard culture medium or osteogenic medium for a total period of 14 days. Osteoblast-derived exosomes were isolated by differential centrifugation: 2000g for 20 minutes to remove cell debris and apoptotic bodies, 10,000g for 30 minutes to remove micro-vesicles, 120,000g for 70 minutes to pellet exosomes. Following the final ultracentrifugation step, the supernatant was removed, the pellet washed in sterile phosphate buffered saline (PBS) and further centrifuged at 120,000g. The resulting pellet was re-suspended in 200 μ L of PBS and the total protein concentration determined using a Pierce BCA protein assay kit (Thermofisher Scientific, <http://www.thermofisher.com>). Particle size distribution was analysed using Dynamic Light Scattering (DLS, Malvern Instruments) and quantitated using resistive pulse sensing (IZON qNano).

20

Atomic Force Microscopy

5 μ L of total exosome suspension was adsorbed onto freshly cleaved mica sheets and left to dry at 4°C overnight. Samples were rinsed with deionised water and left to dry naturally in a flow hood. The presence of exosomes was determined using a NanoWizard II atomic force microscope (JPK Instruments) under tapping mode using soft cantilevers. Exosome topography and height were recorded simultaneously using a scan rate of 0.25Hz. Image processing was performed using JPK SPM 3.1 software.

30

Stem cell culture in the presence of exosomes

hMSCs were seeded in 6 well culture plates (Nunc) at a density of 21×10^3 cells per cm^2 and incubated at 37°C, 5% CO_2 to allow cell attachment. After 24hrs the medium was replaced with exosome-free medium in the presence and absence of osteogenic

35

supplements, 10mM β -glycerophosphate (Sigma) and 50 μ g/mL L-ascorbic acid (Sigma). Exosomal protein was added to each well at a concentration of 10 μ g/mL. Medium changes and exosome replenishment was performed every 48hrs.

5 Flow Cytometry

Prior to use, 4 μ m latex beads were washed twice in MES buffer and centrifuged at 3000g for 20 minutes and resuspended in 100 μ L MES buffer. 12.5 μ g primary CD9 antibody (eBioscience) was added to 30 $\times$ 10⁶ beads and incubated overnight to allow
10 binding. Resulting antibody coated beads were washed and resuspended in a 100 μ L storage buffer containing 0.1% glycine, 0.1% sodium azide in PBS. Exosomes were added to antibody-conjugated beads at 30 μ g per 1 $\times$ 10⁵ and incubated overnight at 4°C. Nonspecific binding sites were blocked by incubating with 200mM glycine for a period of 30 minutes, and subsequently washed. Secondary fluorescent antibodies,
15 CD9- and CD63, were added to exosome-conjugated beads and incubated at room temperature for 40 minutes with gentle agitation. Fluorescently labelled exosomes were washed, resuspended in PBS and analysed using an FACS Aria I flow cytometry (BD Bioscience).

20 Quantitative Real Time Polymerase Chain Reaction

Total RNA was extracted by re-suspending cells in 500 μ L TRI reagent (Invitrogen), according to the manufacturer's instructions. RNA was phase-separated in chloroform (Sigma), and precipitated using isopropanol (Sigma). Total RNA concentration was
25 determined for each sample using a NanoDrop 2000 spectrophotometer (Thermo Scientific). All primers were pre-designed, and purchased as from Sigma-Aldrich (Table. 2) (Kicqstart primers, Sigma). RT-qRT-PCR was performed using Quantifast™ SYBR® Green RT-PCR one-step kit on a ViiA™ 7 Real-Time PCR system (Life Technologies). Dissociation/melting curve analyses were performed to
30 exclude non-specific amplifications. PCR data were normalised to the reference gene ribosomal protein IIB (RPIIB). Relative gene expression levels were calculated from the cycle threshold (C_t) value using the comparative C_t equation ($^{-\Delta\Delta C_t}$) where relative gene expression is calculated as $2^{-\Delta\Delta C_t}$.

35 Alkaline Phosphatase Assay

Cellular ALP levels were quantified using the SensoLyte® pNPP Alkaline Phosphatase Assay Kit according to the manufacturer's instructions. Briefly, cell monolayers were washed twice using 1x assay buffer provided. Cells were detached
5 from the surface of the culture plate in the presence of 200µL permeabilisation buffer using a cell scraper. The resulting cell suspension was collected in a microcentrifuge tube, incubated at 4°C for 10 minutes under agitation, and then centrifuged at 2500g for 10 minutes. Supernatant was transferred to a 96 well plate where it was combined with an equal volume of pNPP substrate. Absorbance was measured at 405nm using a
10 plate reader.

Alizarin Red Staining and Quantification

Calcium-rich deposits were visualised using 40mM alizarin red (Sigma) stain, which
15 was dissolved in acetic acid and adjusted to pH 4.2 using 5M ammonium hydroxide. Bound alizarin red was quantified through acetic acid extraction and colorimetric detection at 405nm (GloMax Multi Plus, Promega, UK).

Micro-XRF mapping of cell cultures

20

Samples for analysis were prepared by transferring mineralised cell monolayers onto an aluminium foil covered glass slide and leaving to dry overnight at room temperature. Maps of calcium and phosphorous across dried cell culture media spot samples were acquired using a Tornado M4 micro-XRF system (Bruker Nano GmbH,
25 Berlin, Germany) fitted with a Rhodium micro focus X-ray tube and a polycapillary lens, with the X-ray tube voltage set to 50 kV and the tube current set to 300 µA. The chamber pressure was lowered to 20 mbar in order to maximise sensitivity to the phosphorus signal. The system was programmed to acquire a map of the whole slide containing all of the media spot samples by rastering the microfocus beam over the
30 slide with a step size of 30 µm and a time per pixel of 30 ms. An XRF spectrum was collected at each pixel and elemental maps generated progressively in real time by gating around the phosphorous $K_{\alpha 1}$ (2.0137 keV) and calcium $K_{\alpha 1}$ (3.692 keV) X-ray fluorescence peaks in the spectra, creating an image where pixel intensity represented X-ray detector pulses per eV at each measurement point on the sample. Maximum
35 pixel intensity for each element was normalised to the peak pulses/eV value for that

element across the whole sample.

RESULTS AND DISCUSSION

5 MC3T3 murine osteoblasts were cultured in the presence of β -glycerophosphate (osteoconductive donor substrate) according to the methods described herein and extracellular vesicles (EVs), in particular exosomes, were shown to be routinely produced (Figure 1). Atomic force microscopy (Figure 1a) and nanoparticle analysis (Figure 1b) show that EVs with an average size of 150nm are produced by osteoblasts
10 when cultured in osteogenic medium. The EVs were characterised in line with published guidelines (Lotvall J. *J Extracell Vesicles* 2014; 3: 26913) for the presence of membrane lipids and the tetraspanin transmembrane protein CD9 using ImageStream flow cytometry (Figure 1c). In addition, internalisation of osteoblast-derived EVs by MSCs is shown in Figure 1d, this is the mechanism of action believed
15 to be used by the extracellular vesicles to induce bone formation (Figure 2).

The data presented in Figure 2 demonstrates that addition of exosomes to human bone marrow-derived stem cell cultures (MSCs) in the presence of an osteoconductive donor substrate significantly enhances the yield and quality of bone formation. The
20 results presented show that the yield and quality of bone formation is well beyond that of the current gold standard, BMP-2. The isolated EVs (10 μ g/mL EVs) were added to MSC cultures in the presence of an exogenous phosphate-donating substrate, beta-glycerophosphate, (EV OST – Figure 2) and mineralisation was induced in the MSC cultures. The mineralisation is demonstrated by a significant increases in alizarin red
25 calcium staining (Figure 2a), the expression of the mineralisation marker protein alkaline phosphatase (Figure 2b), and the presence of biologically analogous calcium and phosphorus mineral (Figure 2c) which was identified using x-ray fluorescence (XRF) elemental mapping. For the first time, this data shows that an exogenous mineral, in this case phosphate, source is critical for EV(exosome)-mediated
30 mineralisation in MSC cultures. This demonstrates that EVs themselves do not contain a source of calcium and phosphorus but rather are a potent combination of biological factors that allow cells to process local exogenous sources of these minerals. The data presented here is the first evidence to demonstrate that osteogenesis in the presence of EVs and a phosphate donating substrate leads to the formation of mineral that is
35 analogous to mature bone. Mineral induced by the current gold standard, BMP-2, was

shown to be of poorer quality compared with EV OST samples, as evidenced by no co-localisation (yellow) of calcium (red) and phosphorus (green) (Figure 2c and 3). The enhanced maturity of the mineral formed using EV OST will reduce risk of secondary fracture and therefore will have considerable clinical and socioeconomic benefits.

5 Proteomic analysis showed more than a four-fold increase in osteopontin and more than a three-fold increase in collagen type-VI (alpha-1, -2, and -3 chains) in EVs isolated from mineralising osteoblast cultures when compared with EVs from non-mineralising osteoblasts (Figure 4). Osteopontin is recognised as a key regulator of mineralisation and collagen type-VI functions in mediating osteoblast interactions

10 during bone formation. The authors anticipate that increases in these proteins are, in part, responsible for their osteogenesis-enhancing effects.

The data presented in Figure 5 demonstrates that extracellular vesicles can be used for the delivery of mineralising elements to enhance bone formation. The figure

15 demonstrates that when administered in the absence of cells the extracellular vesicles act as independent sites for the delivery and uptake of elements critical for mineralisation. As such, they can be used as an acellular source for enhancing bone regeneration.

20 **Delivery of extracellular vesicles**

In order to deliver extracellular vesicles according to the invention an injectable delivery system has been developed. This is an example and other delivery systems are possible. The particular system developed is summarised in Figure 6.

25

First an alginate fluid gel was prepared. A range of fluid gels with different compositions were produced (Figure 7a). A mechanical agitator provided the shear field for the formation of the fluid gel while the alginate solution was undergoing gelation. The cross-linker (CaCl_2) was added slowly close to the impeller to enable the

30 formation of a homogeneous gel phase. After the addition of the cross-linker, gels were kept under mixing conditions for 10 min, and were then sonicated for 15 min to remove trapped air bubbles. The entire gelation process was performed at room temperature, and to guarantee reproducibility of the fluid gel preparation, each composition was made in duplicate. All gels were stored at 4°C for 24 h before being

35 used for rheological studies.

Fluid gel compositions with high similarity to the control were chosen for extracellular vesicles encapsulation. The encapsulation process was carried out by suspending extracellular vesicles (6×10^9 particles /mL) in alginate solution prior to crosslinking.

Alginate microparticles were prepared by suspending extracellular vesicles at the concentration of 6×10^9 particles /mL in sterile 1%w/v alginate solution (1 mL). The microparticles were generated using an encapsulator with the vibrational nozzle technology (A B-395 pro, Buchi, UK) under sterile condition. The generated droplets were incubated in a crosslinking solution (0.1 M CaCl_2) located at 20 cm from the nozzle head for 10 min. The encapsulator parameters were set as follow: nozzle inner diameter=200 μm , frequency=600 Hz, electrode=1200 v, and pump flow rate=40 mL/min. The generated microparticles were then subjected to several washes with PBS to remove any remaining unbound calcium ions.

Confocal microscopy was performed to demonstrate the distribution of extracellular vesicles within these two systems using a lipid dye (FM1-43FX) to stain the phospholipid membrane (Figure 8).

To ensure that the FM1-43FX dye was staining extracellular vesicles a co-localisation experiment was performed by also staining extracellular vesicles with SYTO ENA select (Figure 9). A high correlation of co-location was observed between these two dyes (Pearson's correlation = 0.819 +/- 0.06).

The release of extracellular vesicles from both alginate delivery systems was quantified using Nano Tracking Analysis (NTA) and CD63 ExoELISA (Figure 10). Notably, since extracellular vesicles were incorporated into the cross-linked gel and continuous phase of the fluid gel (Figure 6), the release kinetics compared with the cross-linked microparticles was significantly higher from the fluid gel. This enables agents within the size range of the extracellular vesicles (Figure 10A) to be released from the alginate system, which would not be possible to the same degree within a cross-linked delivery system manufactured from the same material. Thus the use of the fluid gels facilitates a greater release of extracellular vesicles as demonstrated in NTA data (Figure 10B). Notably ELISA data could not be generated for the

extracellular vesicles released from the microparticles since the amounts were below the detectable limits of this assay.

CLAIMS

1. Extracellular vesicles derived from osteoblasts or osteoblast-like cells comprising elevated levels of one or more of an annexin protein, collagen type-VI
5 and/or osteopontin.
2. The extracellular vesicles of claim 1 wherein the level of Annexin 2 is at least 2 fold more than that observed in extracellular vesicles derived from non-mineralising osteoblasts or osteoblast-like cells, and/or the level of collagen type-VI is at least 3
10 fold more than that observed in extracellular vesicles derived from non-mineralising osteoblasts or osteoblast-like cells.
3. A method of producing extracellular vesicles from osteoblasts or osteoblast-like cells wherein the osteoblasts or osteoblast like cells are cultured in a growth
15 medium containing between about 1mM and about 100mM beta-glycerophosphate, and optionally ascorbic acid at a concentration of between about 1µg/ml and about 100µg/ml.
4. The method of claim 3 wherein the osteoblasts or osteoblast like cells are
20 cultured in a growth medium containing between about 8mM and about 15mM beta-glycerophosphate, and optionally ascorbic acid at a concentration of between about 30µg/ml and about 100µg/ml.
5. The pharmaceutical composition comprising extracellular vesicles as defined
25 in claim 1 or 2 or produced by the method of claim 3 or 4.
6. A composition comprising extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate.
- 30 7. The composition of claim 6 wherein the composition is a pharmaceutical composition and further comprises a pharmaceutically acceptable carrier.
8. A method of promoting bone formation/regeneration in a subject at a site in need thereof, the method comprising the steps of:

- (i) obtaining extracellular vesicles obtained from osteoblasts or osteoblast-like cells;
- (ii) administering the extracellular vesicles to a site in a subject where the promotion of bone formation/regeneration is required; wherein the extracellular vesicles are administered with one or more osteoconductive donor substrates.

9. A method of promoting bone formation/regeneration *in vitro*, the method comprising the steps of:

- (i) obtaining extracellular vesicles obtained from osteoblasts or osteoblast-like cells;
- (ii) administering the extracellular vesicles to cells *in vitro*; wherein the extracellular vesicles are administered with one or more osteoconductive donor substrates.

10. The method according to claim 9 wherein the cells are autologous or heterologous stem cells, osteoblasts or osteoblast-like cells.

11. The method of claims 8, 9 or 10 wherein the extracellular vesicles obtained in step (i) were obtained from osteoblasts or osteoblast-like cells cultured in the presence of one or more osteoconductive donor substrate.

12. The method of any of claims 9 to 11 wherein the extracellular vesicles and osteoconductive donor substrate are administered sequentially, simultaneously or separately.

13. A method of treating a bone condition in a subject in need of bone regeneration comprising locally administering sequentially, simultaneously or separately to the subject a therapeutically effective amount of extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate.

14. Use of extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate in the manufacture of a medicament for use in the treatment of a bone condition or in promoting bone growth/regeneration.

15. Extracellular vesicles derived from osteoblasts or osteoblast-like cells for use in the treatment of a bone condition or in promoting bone growth/regeneration, wherein the extracellular vesicles derived from osteoblasts or osteoblast-like cells are intended to be administered with an osteoconductive donor substrate.

5

16. Extracellular vesicles for the use of claim 15 wherein the extracellular vesicles and osteoconductive donor substrate are intended to be administered sequentially, simultaneously or separately.

10 17. Extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate for use in the treatment of a bone condition or in promoting bone growth/regeneration.

15 18. A kit comprising extracellular vesicles derived from osteoblasts or osteoblast-like cells and an osteoconductive donor substrate and instructions for administration to a subject to treat a bone condition and/or to aid in bone growth/regeneration.

19. The kit of claim 18 wherein the extracellular vesicles and osteoconductive donor substrate are provided in the same composition.

20

20. The kit of claim 18 wherein the extracellular vesicles and osteoconductive donor substrate are provided in separate compositions.

21. An implantable device which carries or contains extracellular vesicles and/or
25 an osteoconductive donor substrate.

22. The extracellular vesicles, composition, method, use, kit or device of any preceding claim wherein the extracellular vesicles are exosomes.

30 23. The extracellular vesicles, composition, method, use, kit or device of any preceding claim wherein the osteoconductive donor substrate is a mineral donating substrate.

35 24. The extracellular vesicles, composition, method, use, kit or device of claim 23 wherein the mineral donating substrate comprises calcium, phosphorus, magnesium,

sodium, chloride, pyrophosphate, potassium, fluoride, strontium, zinc, chromium, cobalt, copper, manganese, silicon, carbonate or bicarbonate.

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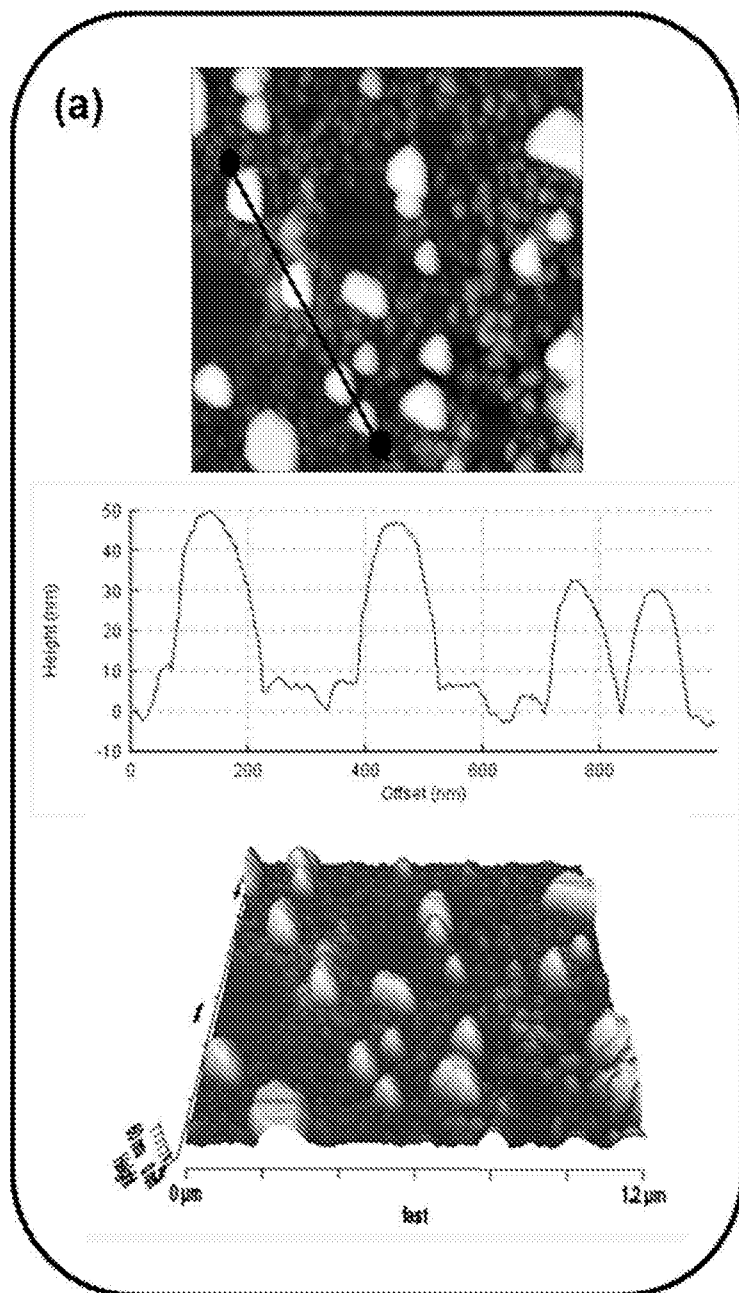


Figure 1

2/17

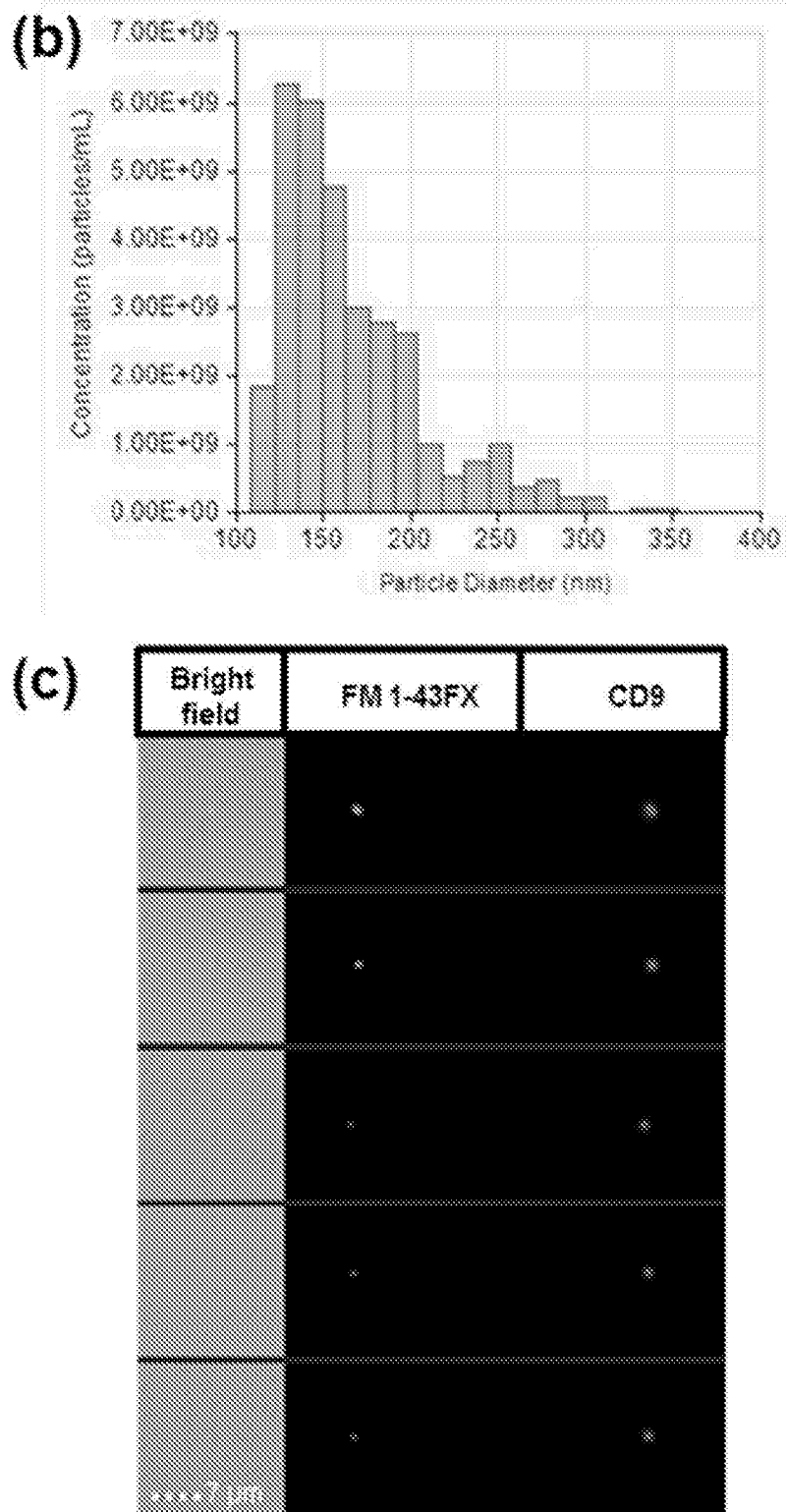


Figure 1 continued

3/17

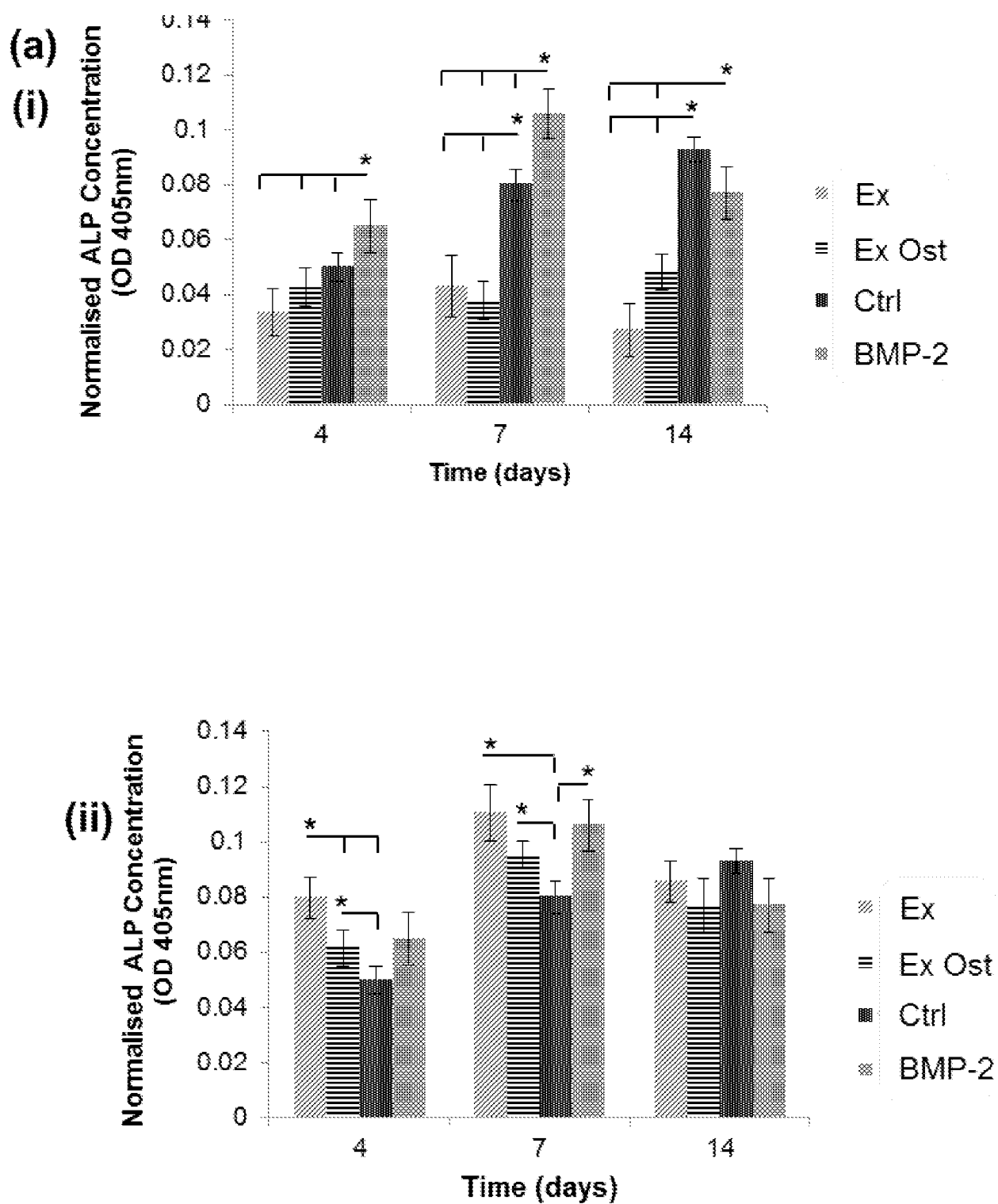


Figure 2

4/17

(b)

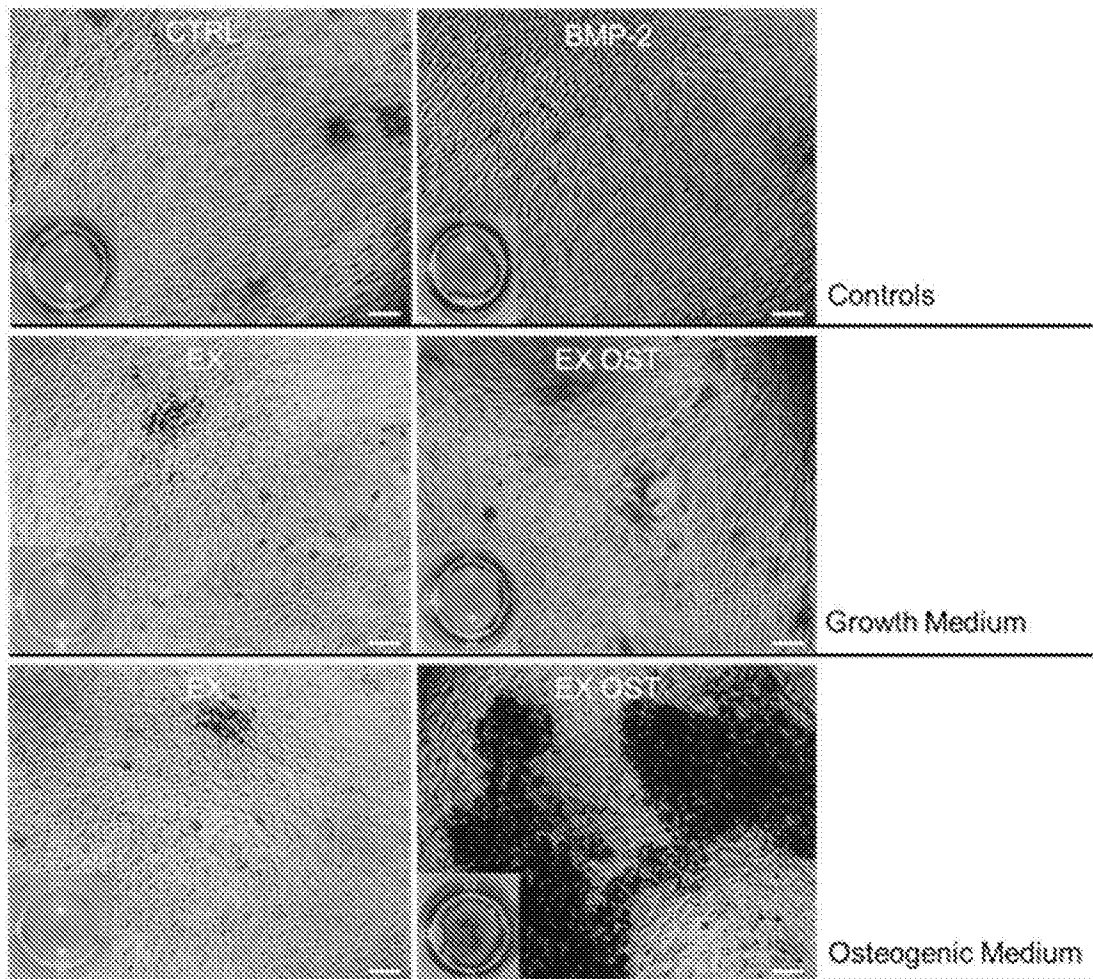


Figure 2 continued

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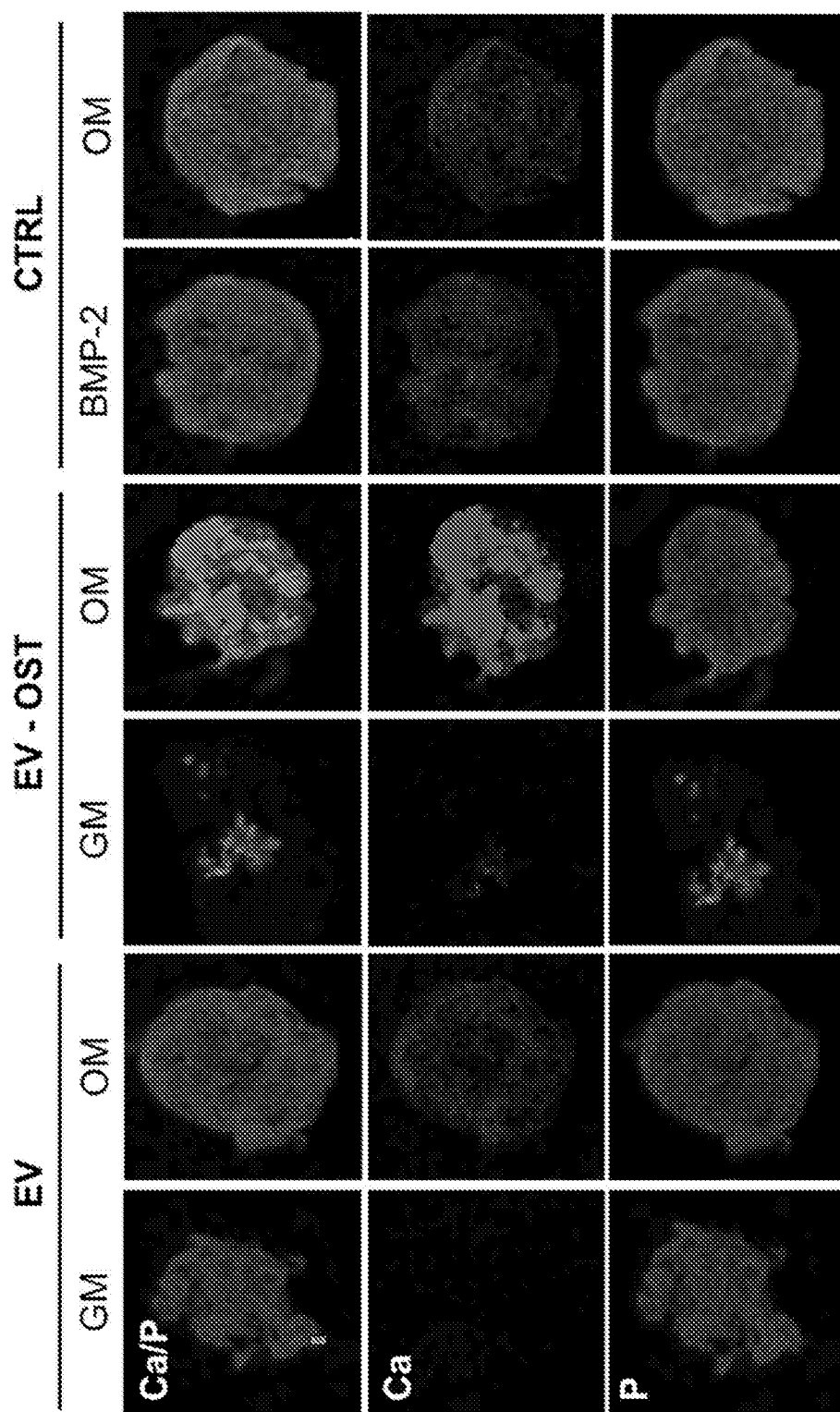


Figure 3

6/17

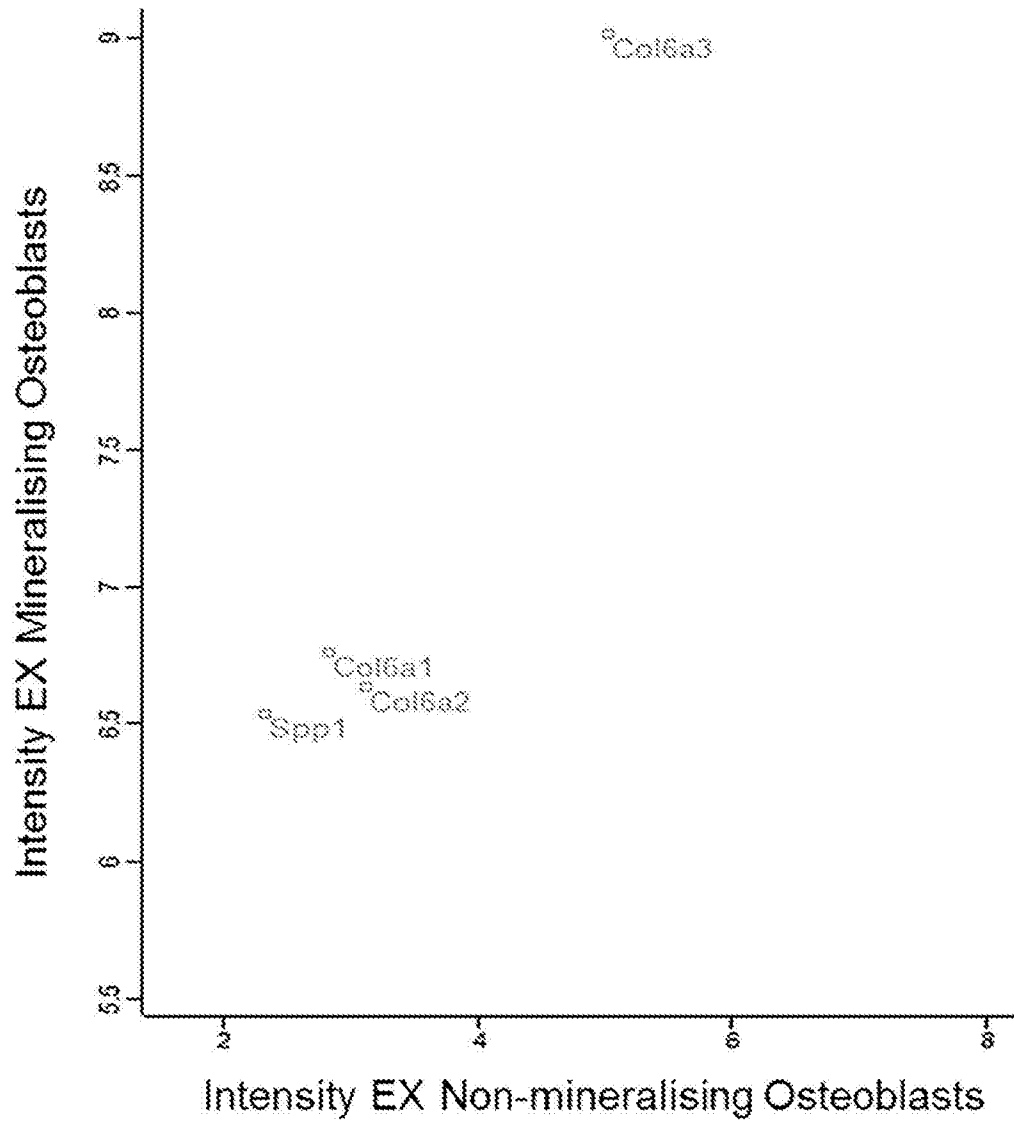


Figure 4

7/17

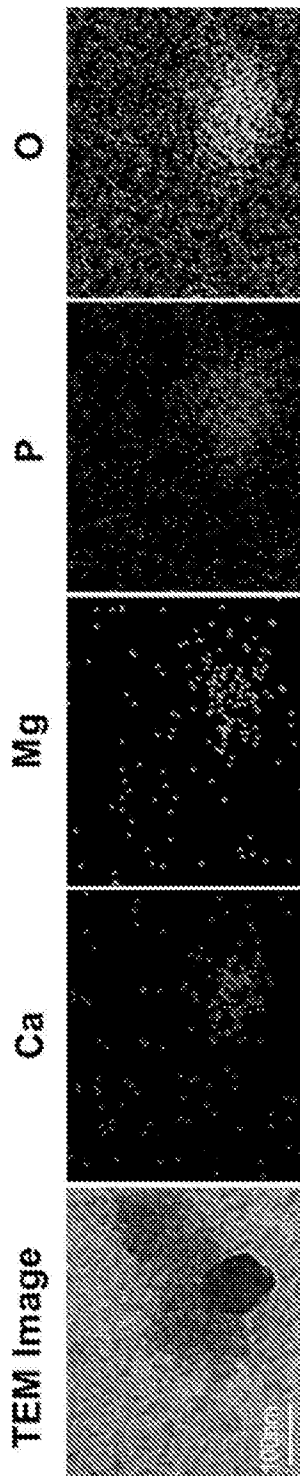


Figure 5

8/17

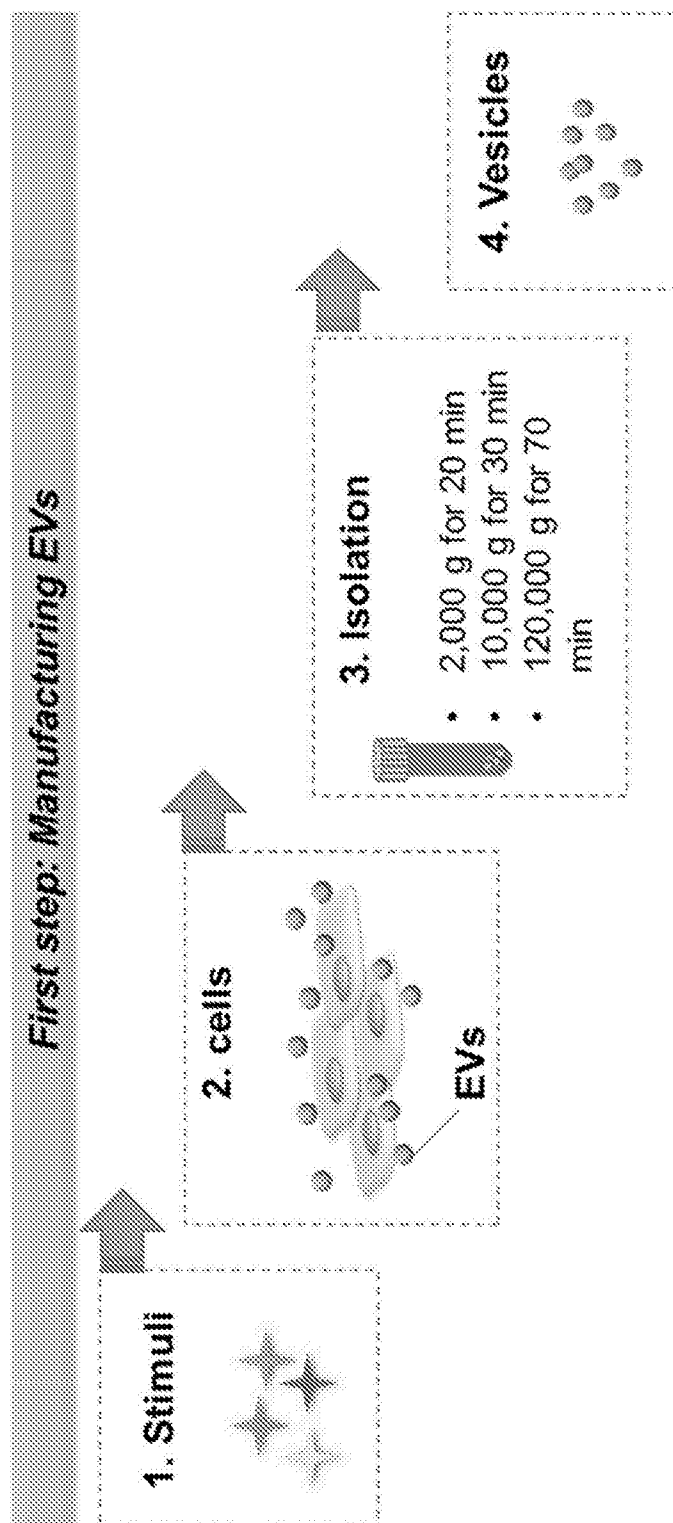


Figure 6

9/17

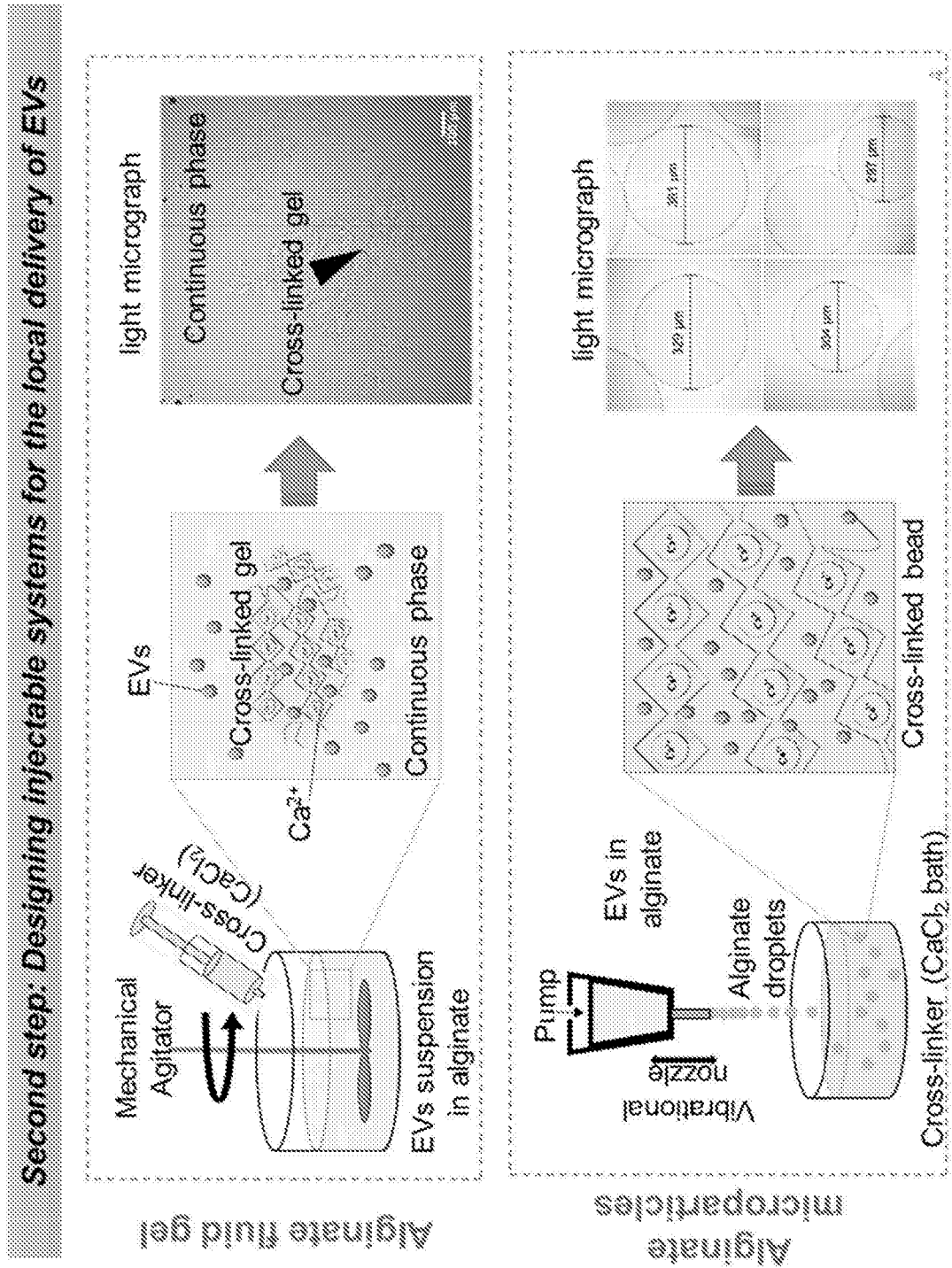


Figure 6 continued

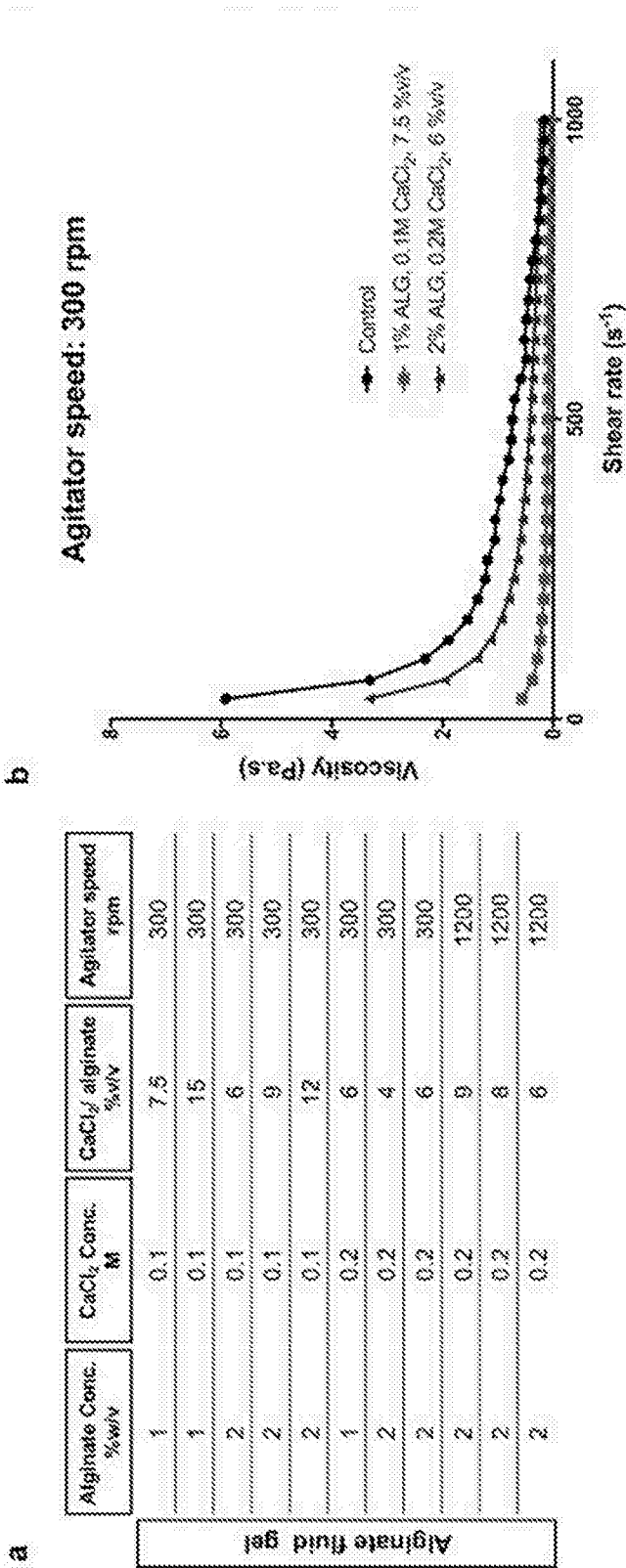


Figure 7

11/17

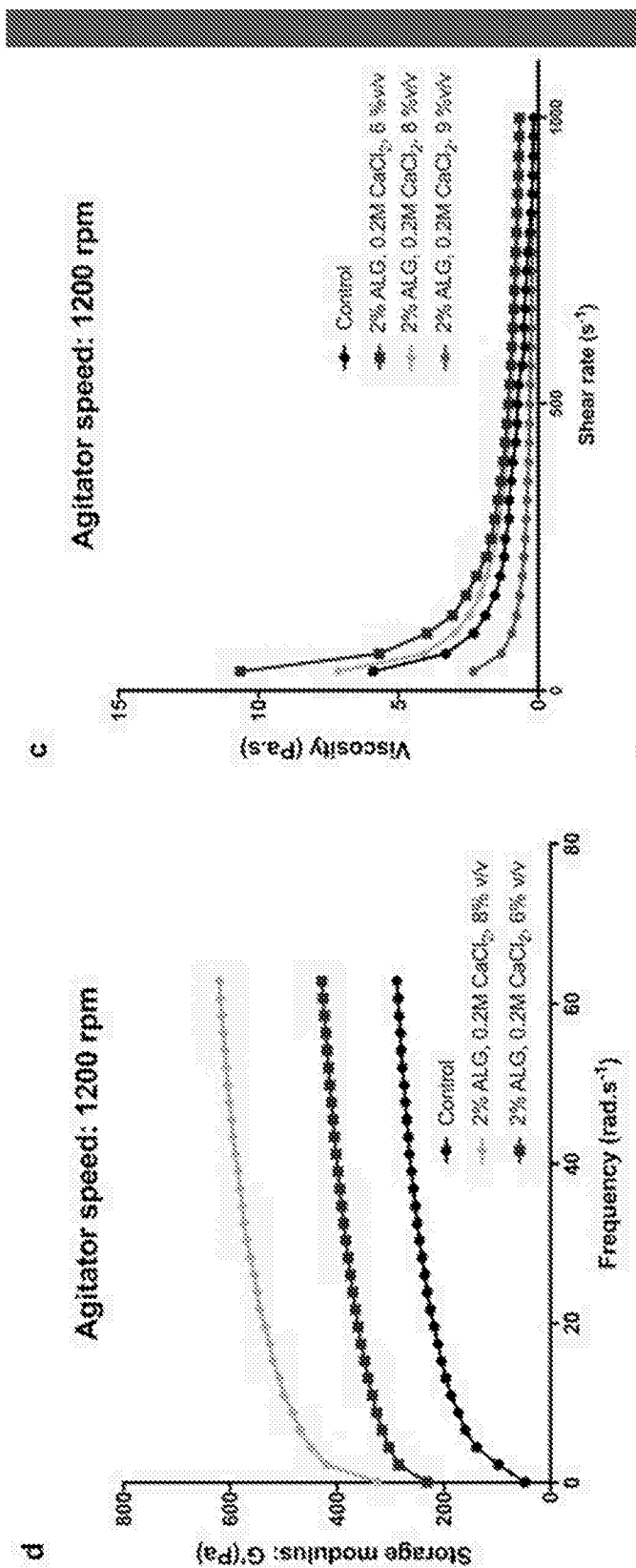


Figure 7 continued

12/17

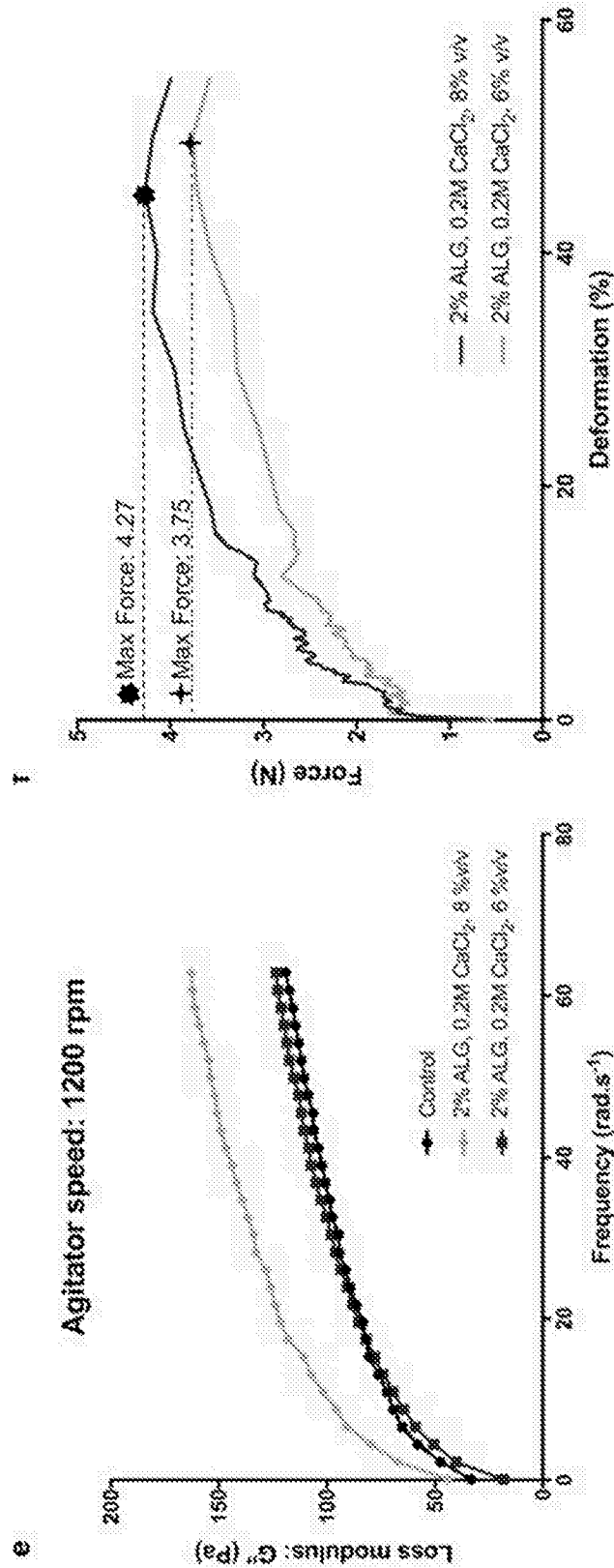
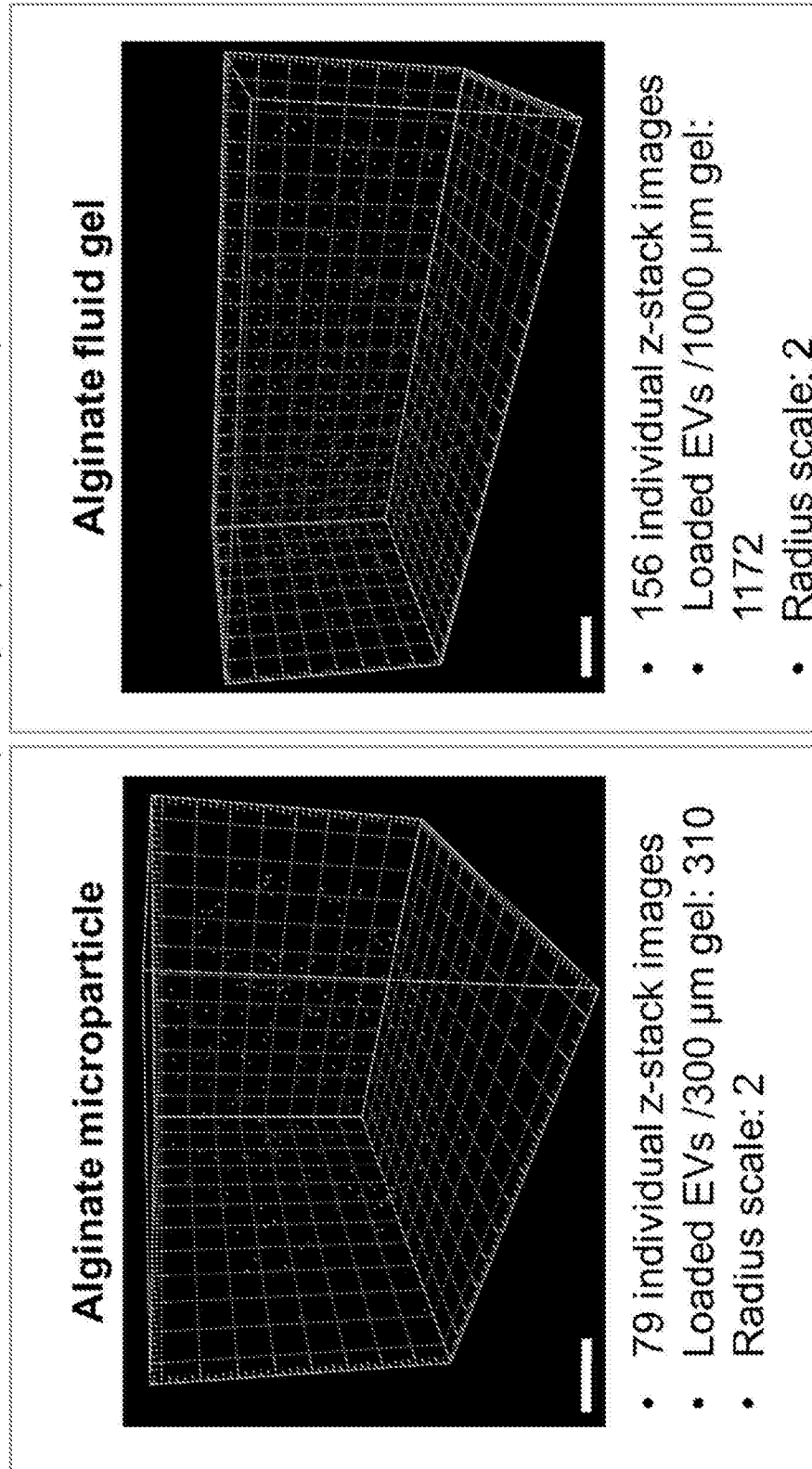


Figure 7 continued

13/17

3D reconstructed confocal micrographs EVs labeled with lipid dye (FM1-43FX)



Scale bars show 100 μm .

Figure 8

14/17

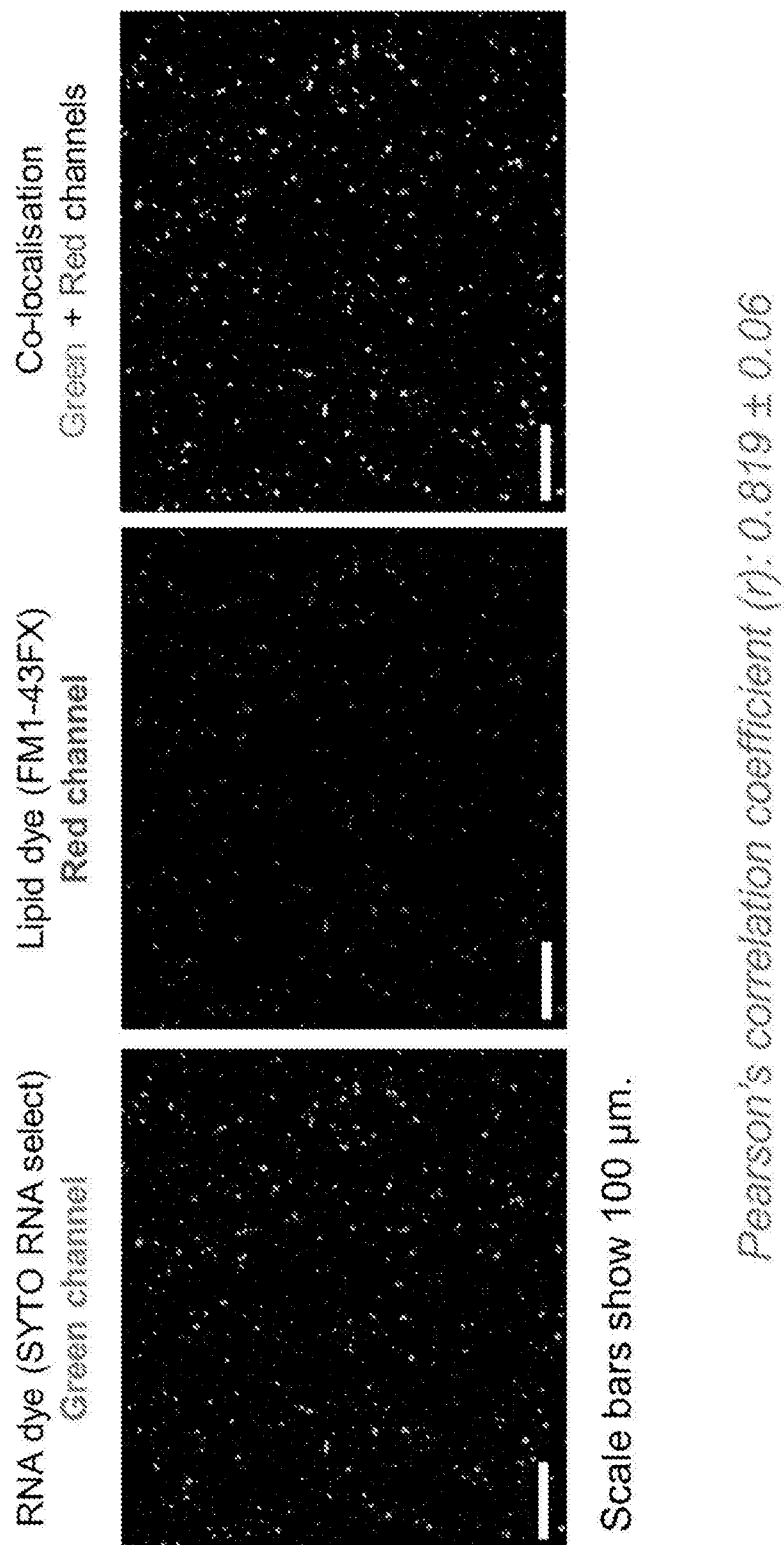


Figure 9

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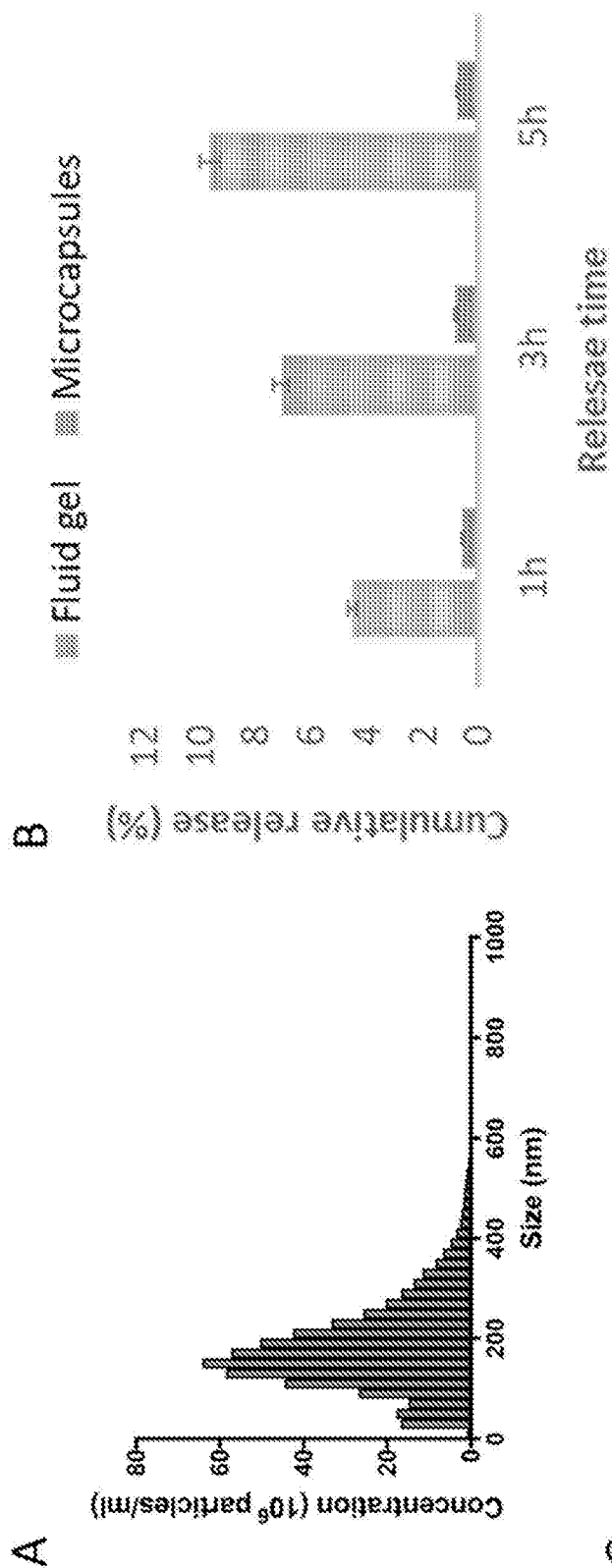


Figure 10

16/17

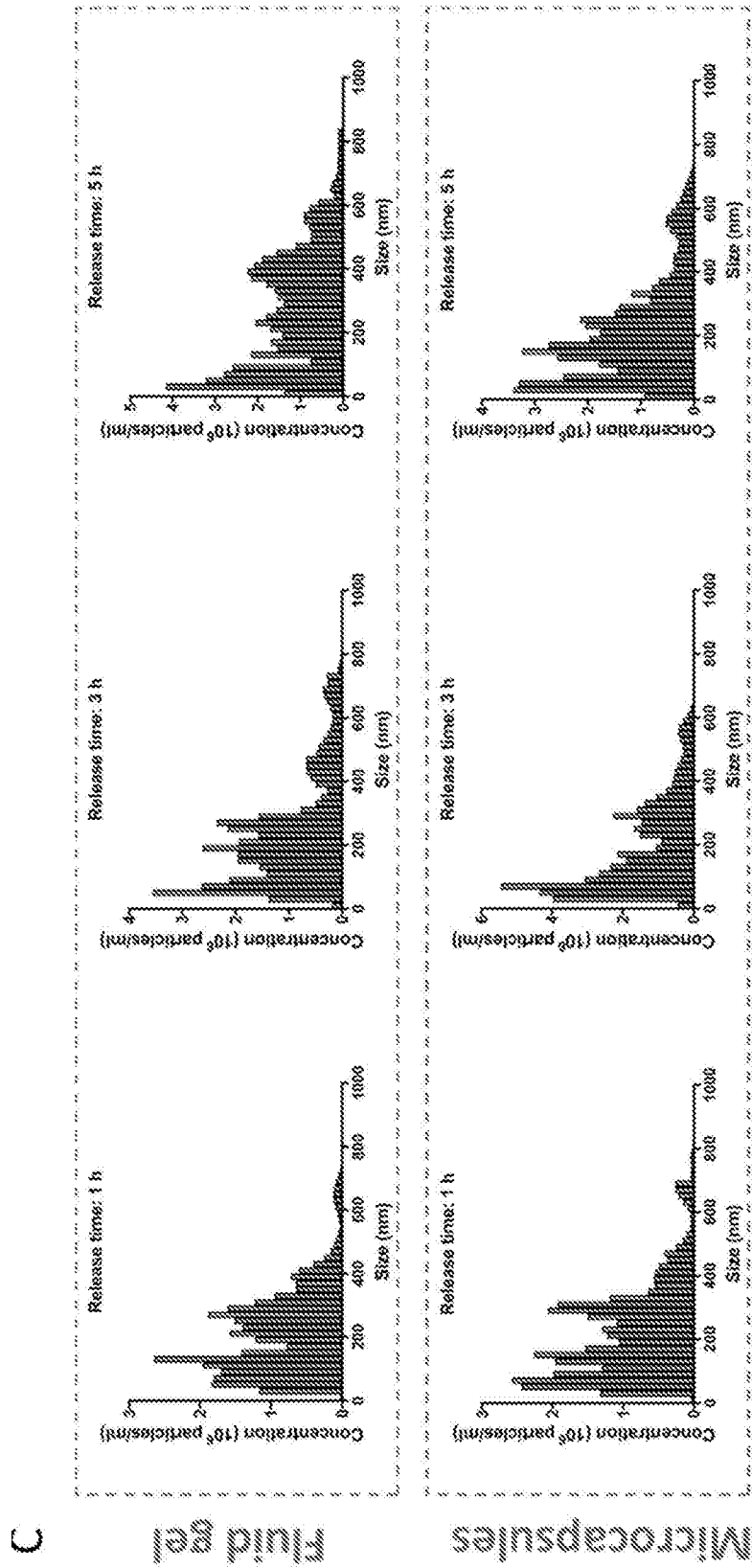


Figure 10 continued

17/7

C continued

Sample	Mean (nm) ± SEM	d 50 (nm) ± SEM
EVs	186 ± 4	169 ± 3
FG-1h*	211 ± 9	202 ± 21
FG-3h	259 ± 38	271 ± 61
FG-5h	267 ± 43	223 ± 60
MP-1h*	222 ± 25	254 ± 49
MP-3h	188 ± 21	140 ± 26
MP-5h	227 ± 43	207 ± 46

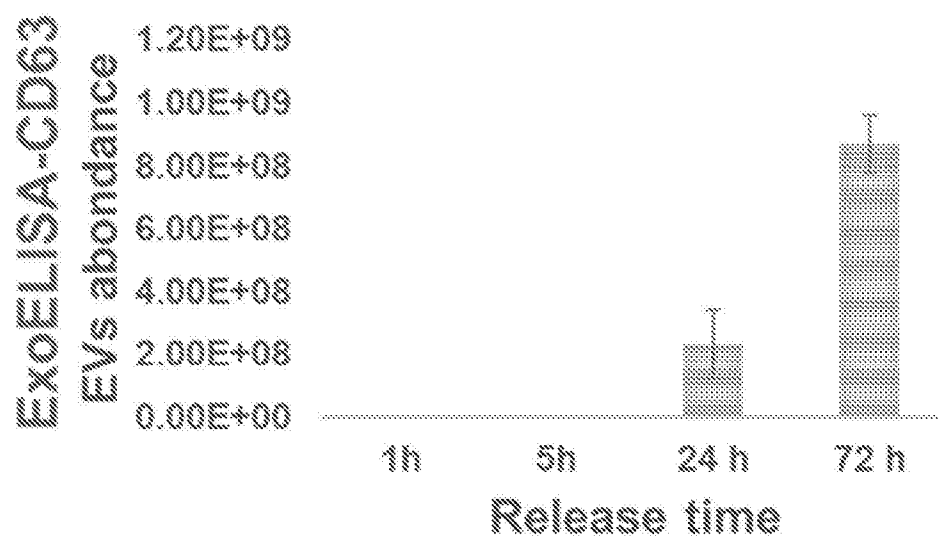
D**Long-term release study**

Figure 10 continued

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2017/053840

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K35/32 A61L27/00 A61P19/08 C12N5/077
ADD.

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)

A61K

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)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2016/201154 A1 (ORTHOCYTE CORP [US]) 15 December 2016 (2016-12-15) page 14, lines 28-30; examples 1,4-5 -----	1-24
X	ANNA CMOCH ET AL: "Matrix vesicles isolated from mineralization-competent Saos-2 cells are selectively enriched with annexins and S100 proteins", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 412, no. 4, 2011, pages 683-687, XP028439129, ISSN: 0006-291X, DOI: 10.1016/J.BBRC.2011.08.025 [retrieved on 2011-08-16] pages 684-685 ----- -/-	1-3,5, 18-20, 22-24

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

☒ See patent family annex.

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2017/053840

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CYRIL THOUVEREY ET AL: "Proteomic characterization of biogenesis and functions of matrix vesicles released from mineralizing human osteoblast-like cells", JOURNAL OF PROTEOMICS, ELSEVIER, AMSTERDAM, NL, vol. 74, no. 7, 8 April 2011 (2011-04-08), pages 1123-1134, XP028387682, ISSN: 1874-3919, DOI: 10.1016/J.JPROT.2011.04.005 [retrieved on 2011-04-15] page 1124 - page 1125 page 1127	1-3,5, 18-20, 22-24
Y	----- ALFORD A I ET AL: "Thrombospondin-2 regulates matrix mineralization in MC3T3-E1 pre-osteoblasts", BONE, PERGAMON PRESS., OXFORD, GB, vol. 46, no. 2, 1 February 2010 (2010-02-01), pages 464-471, XP026864595, ISSN: 8756-3282 [retrieved on 2009-09-08] pages 465,468; figure 7 -----	1-24
Y	----- WO 2013/169202 A1 (BIOMATCELL AB [SE]) 14 November 2013 (2013-11-14) page 9, line 19 - line 30; claim 14; example 1 -----	1-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2017/053840

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