

**A MEMBRANE HOLDING DEVICE FOR PRODUCING A TISSUE
MEMBRANE, A METHOD OF PRODUCING A TISSUE MEMBRANE, AND A
TISSUE MEMBRANE CONSTRUCT**

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TECHNICAL FIELD

The present disclosure relates broadly to a membrane holding device for
10 producing a tissue membrane, a method of producing a tissue membrane, and a
tissue membrane construct.

BACKGROUND

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Cell spheroids have become increasingly used as building blocks for
tissue engineering (TE). This is due to the distinct advantages of cell spheroids
over conventional cellular monolayers, by leveraging on the self-organization,
direct cell-cell interactions and relatively higher cell densities of cell spheroids to
20 create complex and functional tissue structures, which mimic many aspects of
native tissues.

Cell spheroids may be used in a wide range of tissue engineering
applications including cartilage tissue engineering. Cartilage tissue engineering
25 aims to make cartilage *in vitro* from human cells for the treatment of various
orthopaedic problems. The current state-of-the-art cartilage TE favours small cell
spheroids (about 200 μm to 300 μm) as building blocks because its spherical
morphology helps in cartilage development and the small size allows efficient
medium diffusion.

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However, there are problems with current methods of making large
cartilage tissue using cell spheroids.

When assembling spheroids in a large mould, spheroids tend to fuse into a thick structure, thereby impeding medium diffusion, causing formation of a necrotic core and non-uniform cartilage development.

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When embedding spheroids in a large hydrogel structure, medium diffusion is hindered by the hydrogel network. In addition, most hydrogels do not possess sufficient mechanical strength for orthopaedic applications.

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When seeding spheroids into natural/synthetic scaffolds, spheroids still tend to fuse into a thick structure at relatively high seeding densities, causing diffusion issues and non-uniform cartilage development.

When assembling spheroids in a needle array, expensive three-dimensional (3D) printing equipment is required, and the relatively low throughput does not justify the high cost of this technique.

Thus, there is a need for a membrane holding device for producing a tissue membrane, a method of producing a tissue membrane, and a tissue membrane construct, which seek to address or at least ameliorate one of the above problems.

SUMMARY

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In one aspect, there is provided a membrane holding device for producing a tissue membrane, the device comprising, a first plate comprising a plurality of holes; and a second plate comprising a plurality of holes, said second plate configured to be detachably coupled to the first plate; wherein the first and second plates are configured to exert a substantially even compression on a tissue membrane construct held therebetween, when coupled together.

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In one embodiment, the first plate comprises a first grid with a network of grid bars defining the plurality of holes; the second plate comprises a second grid with a network of grid bars defining the plurality of holes; and the first and second grids are configured to be aligned when the first and second plates are coupled together.

In one embodiment, the grid bars have an average thickness falling in the range of from 0.1 mm to 10 mm.

In one embodiment, for each of the first and second plates, the ratio of area occupied by the network of grid bars to the total area of the grid falls in the range of from 1% to 60%.

In one embodiment, each hole in the plurality of holes has dimensions of 5 mm by 5 mm to 50 mm by 50 mm.

In one embodiment, the plurality of holes have substantially the same dimensions and are spaced apart in a regular manner.

In one embodiment, the membrane holding device further comprises a first membrane with one side configured to contact the first plate and an opposite side configured to interface with a layer of cell spheroids; and a second membrane with one side configured to contact the second plate and an opposite side configured to interface with the layer of cell spheroids.

In one embodiment, the first and second membranes are in the form of meshes formed using threads having an average diameter of less than 200 μm .

In one embodiment, the membrane holding device further comprises one or more coupling members configured for facilitating coupling of the first plate to the second plate, wherein the one or more coupling members are further

configured to allow adjustable control of the compression applied to the tissue membrane construct.

5 In one embodiment, the membrane holding device is stackable with a plurality of membrane holding devices to facilitate production of a plurality of tissue membranes.

10 In one embodiment, the membrane holding device is configured to be integrated into a bioreactor system for facilitating transport of substances into and out of the tissue membrane construct.

15 In another aspect, there is provided a method of producing a tissue membrane, the method comprising, providing a membrane holding device comprising a first plate and a second plate configured to be detachably coupled to the first plate; assembling a tissue membrane construct comprising a layer of cell spheroids disposed between a first membrane and a second membrane, such that the tissue membrane construct is held in position between the first and second plates of the membrane holding device; and maintaining the membrane holding device with the tissue membrane construct under culture conditions to produce the tissue membrane.

In one embodiment, the method further comprises a step of seeding cell spheroids to form the layer of cell spheroids.

25 In one embodiment, the seeding step comprises, placing the first membrane on the first plate; placing the second plate relative to the first plate such that the first membrane is disposed between the first and second plates; depositing cell spheroids onto the first membrane via a plurality of holes provided in the second plate to form the layer of cell spheroids; removing the second plate; 30 positioning the second membrane over the layer of cell spheroids such that the layer of cell spheroids is disposed between the first and second membranes; and coupling the first and second plates to each other.

In one embodiment, the first and second membranes comprise a plurality of pores, and the plurality of pores have an average pore size which is smaller than an average diameter of the cell spheroids.

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In one embodiment, the plurality of pores of the first and second membranes have an average diameter falling in the range of from 0.1 μm to 500 μm .

10 In one embodiment, the cell spheroids have an average diameter falling in the range of from 50 μm to 2000 μm .

In one embodiment, the cell spheroids comprise from 100 cells to 500,000 cells per spheroid.

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In one embodiment, the cell spheroids are seeded at a density falling in the range from 500 to 10,000 cell spheroids per cm^2 .

20 In one embodiment, the tissue membrane has an average thickness falling in the range of from 50 μm to 2000 μm .

In yet another aspect, there is provided a tissue membrane construct comprising, a first membrane comprising a plurality of pores; a second membrane comprising a plurality of pores; and a layer of cell spheroids disposed between the first and second membranes.

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In one embodiment, the tissue membrane construct is stackable with one or more additional tissue membrane constructs to form a thicker tissue construct.

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DEFINITIONS

The term "bioactive" as used herein refers to materials that interact with the surrounding biological system. In various embodiments, a bioactive material
5 comprises compounds or entities that alter, promote, speed, prolong, inhibit, activate, or otherwise affect biological or chemical events in a subject (e.g., a human).

The term "biocompatible" as used herein refers to materials that are well-
10 tolerated by a biological system. In various embodiments, the term "biocompatible" refers to the ability of a material to perform its intended function without inducing significant inflammatory response, immunogenicity, or cytotoxicity to cells, tissues, or organs.

15 The term "bioinert" as used herein refers to any material that does not elicit a response from a host or interact when introduced to a biological tissue.

The term "cell spheroids" or "spheroids" as used herein refers to cell aggregates that are self-assembling in an environment that prevents attachment
20 to a flat surface. Spheroids are substantially spherical cellular units that are generally cultured as free-floating aggregates. Multi-cellular spheroids are formed as a 3D structure with dense cell-cell/cell-extracellular matrix interactions and have been widely utilized as implantable therapeutics and various *ex vivo* tissue models in tissue engineering. In various embodiments, cell spheroids are
25 generated from expanded cells by common methods in the art such as using a microwell plate, a hanging drop assay, a gel embedding/hydrogel embedding assay, a magnetic levitation assay, a spinner culture/spinner flask, a microfluidic chip, a bioreactor, a pellet culture, a bioprinting process, and the like.

30 The term "preventing" and/or "reducing the severity of symptoms" as used herein refer to a process of delaying the onset, reducing the severity of symptoms, reducing and/or preventing weight loss, preventing death, inhibiting

deterioration, inhibiting further deterioration, and/or ameliorating at least one sign or symptom of a disease.

The term "subject" as used herein includes patients and non-patients. The
5 term "patient" refers to individuals suffering or are likely to suffer from a medical condition, while "non-patients" refer to individuals not suffering and are likely to not suffer from the medical condition. "Non-patients" include healthy individuals, non-diseased individuals and/or an individual free from the medical condition. The term "subject" includes humans and animals. Animals may include, but are
10 not limited to, mammals (for example non-human primates, canine, murine, leporid, bovine, porcine, and the like), and the like. "Murine" refers to any mammal from the family Muridae, such as mouse, rat, and the like. "Leporid" refers to any mammal from the family Leporidae, such as hare, rabbit, and the like.

15 The term "tissue membrane" as used herein refers to a thin layer or sheet of cells or tissues that covers or lines an exterior of a body (such as, skin), organs (such as, pericardium), internal passageways that lead to the exterior of the body (such as abdominal mesenteries), and linings of moveable joint cavities. The two
20 broad categories of tissue membranes in the body are i) connective tissue membranes which include synovial membranes and ii) epithelial membranes which include mucous membranes, serous membranes, and the cutaneous membrane, in other words, the skin.

The term "treating", "treat" and "therapy", and synonyms thereof refer to
25 both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) a medical condition, which includes, but is not limited to, diseases, symptoms, and disorders. A medical condition also includes a body's response to a disease or disorder, e.g., inflammation. Those in need of such treatment include those already with a medical condition as well as
30 those prone to getting the medical condition or those in whom a medical condition is to be prevented.

The term “substrate” as used herein is to be interpreted broadly to refer to any supporting structure.

The term “layer” when used to describe a first material is to be interpreted
5 broadly to refer to a first depth of the first material that is distinguishable from a
second depth of a second material. The first material of the layer may be present
as a continuous film, as discontinuous structures or as a mixture of both. The
layer may also be of a substantially uniform depth throughout or varying depths.
Accordingly, when the layer is formed by individual structures, the dimensions of
10 each of individual structure may be different. The first material and the second
material may be same or different and the first depth and second depth may be
same or different.

The term “continuous” when used to describe a film or a layer is to be
15 interpreted broadly to refer to a film or a layer that is substantially without gaps or
holes or voids across the film or layer. In this regard, a continuous film or a
continuous layer is also intended to include a film or a layer that may have trivial
gaps or holes or voids that may not appreciably affect the desired properties of
the film or the layer.

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The term “micro” as used herein is to be interpreted broadly to include
dimensions from about 1 micron to about 1000 microns.

The term “nano” as used herein is to be interpreted broadly to include
25 dimensions less than about 1000 nm.

The term “particle” as used herein broadly refers to a discrete entity or a
discrete body. The particle described herein can include an organic, an inorganic
or a biological particle. The particle used described herein may also be a macro-
30 particle that is formed by an aggregate of a plurality of sub-particles or a fragment
of a small object. The particle of the present disclosure may be spherical,
substantially spherical, or non-spherical, such as irregularly shaped particles or

ellipsoidally shaped particles. The term “size” when used to refer to the particle broadly refers to the largest dimension of the particle. For example, when the particle is substantially spherical, the term “size” can refer to the diameter of the particle; or when the particle is substantially non-spherical, the term “size” can refer to the largest length of the particle.

The terms “coupled” or “connected” as used in this description are intended to cover both directly connected or connected through one or more intermediate means, unless otherwise stated.

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The term “associated with”, used herein when referring to two elements refers to a broad relationship between the two elements. The relationship includes, but is not limited to a physical, a chemical or a biological relationship. For example, when element A is associated with element B, elements A and B may be directly or indirectly attached to each other, or element A may contain element B or vice versa.

The term “adjacent” used herein when referring to two elements refers to one element being in close proximity to another element and may be but is not limited to the elements contacting each other or may further include the elements being separated by one or more further elements disposed therebetween.

The term “and/or”, e.g., “X and/or Y” is understood to mean either “X and Y” or “X or Y” and should be taken to provide explicit support for both meanings or for either meaning.

Further, in the description herein, the word “substantially” whenever used is understood to include, but not restricted to, “entirely” or “completely” and the like. In addition, terms such as “comprising”, “comprise”, and the like whenever used, are intended to be non-restricting descriptive language in that they broadly include elements/components recited after such terms, in addition to other components not explicitly recited. For example, when “comprising” is used,

reference to a “one” feature is also intended to be a reference to “at least one” of that feature. Terms such as “consisting”, “consist”, and the like, may in the appropriate context, be considered as a subset of terms such as “comprising”, “comprise”, and the like. Therefore, in embodiments disclosed herein using the

5 terms such as “comprising”, “comprise”, and the like, it will be appreciated that these embodiments provide teaching for corresponding embodiments using terms such as “consisting”, “consist”, and the like. Further, terms such as “about”, “approximately” and the like whenever used, typically means a reasonable variation, for example a variation of +/- 5% of the disclosed value, or a variance

10 of 4% of the disclosed value, or a variance of 3% of the disclosed value, a variance of 2% of the disclosed value or a variance of 1% of the disclosed value.

Furthermore, in the description herein, certain values may be disclosed in a range. The values showing the end points of a range are intended to illustrate

15 a preferred range. Whenever a range has been described, it is intended that the range covers and teaches all possible sub-ranges as well as individual numerical values within that range. That is, the end points of a range should not be interpreted as inflexible limitations. For example, a description of a range of 1% to 5% is intended to have specifically disclosed sub-ranges 1% to 2%, 1% to 3%,

20 1% to 4%, 2% to 3% etc., as well as individually, values within that range such as 1%, 2%, 3%, 4% and 5%. It is to be appreciated that the individual numerical values within the range also include integers, fractions and decimals. Furthermore, whenever a range has been described, it is also intended that the range covers and teaches values of up to 2 additional decimal places or

25 significant figures (where appropriate) from the shown numerical end points. For example, a description of a range of 1% to 5% is intended to have specifically disclosed the ranges 1.00% to 5.00% and also 1.0% to 5.0% and all their intermediate values (such as 1.01%, 1.02% ... 4.98%, 4.99%, 5.00% and 1.1%, 1.2% ... 4.8%, 4.9%, 5.0% etc.,) spanning the ranges. The intention of the above

30 specific disclosure is applicable to any depth/breadth of a range.

Additionally, when describing some embodiments, the disclosure may have disclosed a method and/or process as a particular sequence of steps. However, unless otherwise required, it will be appreciated that the method or process should not be limited to the particular sequence of steps disclosed. Other
5 sequences of steps may be possible. The particular order of the steps disclosed herein should not be construed as undue limitations. Unless otherwise required, a method and/or process disclosed herein should not be limited to the steps being carried out in the order written. The sequence of steps may be varied and still remain within the scope of the disclosure.

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Furthermore, it will be appreciated that while the present disclosure provides embodiments having one or more of the features/characteristics discussed herein, one or more of these features/characteristics may also be disclaimed in other alternative embodiments and the present disclosure provides
15 support for such disclaimers and these associated alternative embodiments.

DESCRIPTION OF EMBODIMENTS

20 Exemplary, non-limiting embodiments of a membrane holding device for producing a tissue membrane, a method of producing a tissue membrane, and a tissue membrane construct are disclosed hereinafter.

Membrane holding device

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In various embodiments, there is provided a membrane holding device for producing a tissue membrane, the device comprising, a first part e.g., first plate comprising a plurality of holes/openings/apertures; and a second part e.g., second plate comprising a plurality of holes/openings/apertures, said second
30 plate configured to be detachably coupled to the first plate; wherein the first and second plates are configured to exert a substantially even compression on a tissue membrane construct held therebetween, when coupled together.

In various embodiments, the term “membrane holding device” or “membrane holder” as used herein describes the same component and may therefore be used interchangeably. In various embodiments, the first plate may be a bottom plate, and the second plate may be a top plate, and vice versa, depending on how the plates are orientated. For ease of description, the first plate is described as the bottom plate and the second plate is described as the top plate.

In various embodiments, the membrane holding device is configured to facilitate formation of a tissue membrane with the desired size, shape, and thickness, in culture. In various embodiments, the membrane holding device may be made to any size and shape to accommodate the size and shape of the tissue membrane. In various embodiments, the tissue membrane is formed from the tissue membrane construct. In various embodiments, the tissue membrane construct may comprise two or more membranes/meshes and at least one layer of cell spheroids (alternatively referred to as “cell spheroid layer”) disposed between at least one adjacent pair of the two or more membranes, e.g., between any two adjacent membranes. In various embodiments, the membrane holding device is configured to distribute compressive forces on the tissue membrane construct and fabricate a relatively thin cell spheroid layer on the meshes. Without the membrane holding device, cell spheroids tend to clump together even when spread evenly between the meshes.

In various embodiments, the membrane holding device may be used to facilitate formation of a tissue membrane of any tissue that can be tissue-engineered using cell spheroids as building blocks. In various embodiments, the tissues that can be tissue-engineered using cell spheroids include hard and soft tissues. Examples of tissues include, but are not limited to, cartilage, bone, muscle, pancreas, kidney, heart, neural, skin, liver, and vascular tissues. In various embodiments, the tissue membranes may be used for research (e.g., as tissue models for drug testing) or for therapeutic treatment (e.g., as implants). For

example, a hypertrophic cartilage tissue membrane may be used for bone fracture healing.

In various embodiments, the membrane holding device may be stackable
5 with a plurality of membrane holding devices to facilitate production of a plurality
of tissue membranes. In various embodiments, the membrane holding device
may be configured to be integrated into a bioreactor system for facilitating
transport of substances (e.g., by medium perfusion) into and out of the tissue
membrane construct. In various embodiments, a dynamic culture technique using
10 the bioreactor system may further improve medium perfusion into the tissue
membranes as compared to a static culture technique. Examples of bioreactor
systems include, but are not limited to, perfusion bioreactor and spinner flask. For
example, multiple membrane holding devices may be cultured in a larger vessel
or a bioreactor system, such as a perfusion bioreactor or a spinner flask in a
15 stacked manner, such that medium can flow through the plurality of holes from
one end to the other end of the stacked membrane holding devices. In various
embodiments, the membrane holding device and method of producing the tissue
membrane using the membrane holding device may be used to scale up tissue
manufacturing, to produce biomaterials, matrices, or cell-derived materials.

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In various embodiments, the membrane holding device is simple to
assemble and disassemble and allows easy handling of the tissue membrane
construct and tissue membrane for microscopic imaging and various *in vitro*
assays.

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In various embodiments, the membrane holding device may be made of a
hard and/or bioinert material. Examples of suitable materials include, but are not
limited to, metal (e.g., stainless steel, aluminum, gold, titanium), hard plastic (e.g.,
polylactic acid (PLA), polyetheretherketone (PEEK), acrylonitrile butadiene
30 styrene (ABS), polymethyl methacrylate (PMMA)), and the like.

In various embodiments, the membrane holding device may be manufactured using methods known in the art including, but not limited to, machining, 3D printing, molding, casting, and the like.

5 Plates

In various embodiments, the first and second plates are configured to be aligned when coupled to each other. In various embodiments, the first and second plates may have the same shape, e.g., square, rectangular, polygonal, oval or
10 circular/disc shape. In various embodiments, the first and second plates may have similar dimensions to facilitate alignment, e.g., having the same shape, same diameter, length or width. In various embodiments, the first and second plates are simple to assemble and disassemble and allow easy handling of the tissue membrane construct and tissue membrane for microscopic imaging and
15 various *in vitro* assays.

In various embodiments, the first and second plates are configured to immobilize the tissue membrane construct in a fixed position when the tissue membrane construct is held therebetween. In various embodiments, the first and
20 second plates are configured to maintain the shape of the tissue membrane construct when held therebetween. In various embodiments, the first and second plates each comprises a first surface, e.g., exterior surface, and a second surface, e.g., interior surface, disposed opposite the first surface. In various embodiments, the interior surfaces of the first and second plates are configured
25 to face each other when the first and second plates are coupled to each other. In various embodiments, the first and/or second surface of the plates may be substantially planar. In various embodiments, the interior surfaces of the first and second plates may be substantially planar/flat. In various embodiments, the interior surfaces of the first and second plates may be substantially smooth. The
30 substantially planar surfaces may facilitate the application of a substantially even distribution of compressive force on the tissue membrane construct.

In various embodiments, the membrane holding device is configured to maximize medium perfusion and/or medium diffusion in culture. In various embodiments therefore, the first and second plates are configured to assemble the cell spheroid layer into a relatively thin layer, thereby solving the medium diffusion problem in larger tissues. In various embodiments therefore, the plurality of holes disposed in the first and second plates provide access of the tissue membrane construct to medium. In various embodiments, the plurality of holes may have one or more suitable shapes including, but not limited to, circles, ovals, triangles, squares, rectangles, hexagons, pentagons, octagons or any other suitable polygonal shapes. In various embodiments, the plurality of holes in each plate may have the same shape or a combination of different shapes. In various embodiments, the plurality of holes in the first and second plates may have the same size or a combination of different sizes. In various embodiments, the plurality of holes may have different dimensions or substantially identical dimensions. In various embodiments, the plurality of holes may be spaced apart in a regular manner. In various embodiments, the plurality of holes in the first plate are configured to be substantially aligned with the plurality of holes in the second plate when the two plates are coupled together. In other words, each hole in the first plate is configured to be substantially aligned with a corresponding hole of the same shape and size in the second plate, to facilitate movement of substances e.g., flow of medium through both holes.

In various embodiments, the plurality of holes in the first and second plates may facilitate seeding of cells, e.g., cell spheroids onto a membrane of the tissue membrane construct. In various embodiments, the second (top) plate of the membrane holding device may be used to seed cell spheroids evenly during assembly and to easily adjust the size, shape and thickness of the tissue membrane construct. In various embodiments, cell spheroids may be seeded onto a membrane via one or more holes/chambers in the second (top) plate. In various embodiments, cell spheroids may be seeded onto a membrane via all the holes in the second (top) plate. In various embodiments, cell spheroids may be seeded onto a membrane via selected holes in the second (top) plate. In various

embodiments, different densities of cell spheroids (i.e., number of spheroids per cm^2 of a membrane) may be seeded across the holes in the second (top) plate. In various embodiments, the number of holes filled with spheroids and their location may determine the size and shape of the tissue membrane. In various
5 embodiments, the spheroid density in each hole may determine the thickness of the tissue membrane. Accordingly, this allows control of the size, shape, and thickness of the resulting tissue membrane.

In various embodiments, the first plate may comprise a first grid with a
10 network of grid bars defining the plurality of holes and the second plate may comprise a second grid with a network of grid bars defining the plurality of holes. In various embodiments, the network of grid bars may be arranged to intersect with one another to define the plurality of holes. For example, the network of grid bars may comprise evenly spaced, intersecting horizontal and vertical grid bars,
15 forming square- or rectangular-shaped holes. In various embodiments, the grids may be designed with features that allow a suitable and substantially even compression to be applied on the tissue membrane construct and to allow maximal medium perfusion and/or diffusion.

20 In various embodiments, the first and second grids are configured to be aligned when the first and second plates are coupled together. In various embodiments, the grid bars may be configured to have substantially flat/planar surfaces for contacting the membranes of the tissue membrane construct. In various embodiments, the aligning grids may advantageously press firmly and
25 evenly on the membranes of the tissue membrane construct to straighten the membranes, thus constraining the cell spheroids held between the membranes into a relatively thin tissue layer.

In various embodiments, the plurality of holes in the first grid are
30 configured to be substantially aligned with the plurality of holes in the second grid when the first and second plates are coupled to each other. In various embodiments, each pair of aligning holes (i.e., a hole from the first grid and a

corresponding hole from the second grid) form a chamber enclosing a portion of the two or more membranes held between the first and second plates. In various embodiments, each chamber provides access to a portion of the two or more membranes held between the first and second plates. In various embodiments, the plurality of holes may be arranged in an array. In various embodiments, the plurality of holes may be configured to have the same dimensions, e.g., diameter, length, breadth, and area.

In various embodiments, the plates e.g., grids are dimensioned to balance having sufficient stiffness for applying compressive forces on the tissue membrane construct held between the plates, while maintaining sufficient space to facilitate access to the tissue membrane construct and to facilitate movement of substances across the plurality of holes in the plates.

In various embodiments, the grid bars may have an average thickness falling in the range of from about 0.1 mm to about 10 mm. In various embodiments, the grid bar may have a thickness falling in the range with start and end points selected from the following group of numbers: 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75, 5, 5.25, 5.5, 5.75, 6, 6.25, 6.5, 6.75, 7, 7.25, 7.5, 7.75, 8, 8.25, 8.5, 8.75, 9, 9.25, 9.5, 9.75 and 10 mm. In some embodiments, the grid bar has a thickness falling in the range of from about 0.1 mm to about 3 mm. In some embodiments, the grid bar has a thickness of about 1 mm.

In various embodiments, for each of the first and second plates, the ratio of area occupied by the network of grid bars to the total area of the grid may fall in a range of from about 1% to about 60%. In various embodiments, the ratio of area occupied by the network of grid bars to the total area of the grid may fall in the range with start and end points selected from the following group of numbers: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, and 60%. In some

embodiments, the ratio of the grid bar area to the total grid area is about 15% to about 25%.

Without wishing to be bound by theory, a ratio of the grid bar area to the total grid area of about 15% to about 25% has been found to be optimal for the compression of the grids on the membranes/meshes and for maximum medium perfusion and/or diffusion. The grid bars ensure an even compression to keep the meshes flat and prevent mesh bulging during cells/cell spheroid fusion.

In various embodiments, the plurality of holes may be configured to have a hole size that facilitates easy handling, e.g., ease of seeding cell spheroids. In various embodiments, the plurality of holes may be configured to have a hole size that facilitates imaging of a tissue membrane construct held therein. In various embodiments, the plurality of holes may be configured to have a hole size that facilitates performing of *in vitro* assays on a tissue membrane construct held therein.

In various embodiments, each hole in the plurality of holes may have dimensions (width by length) of about 5 mm by 5 mm to about 50 mm by 50 mm. In various embodiments, the hole may have a dimension of about 5 mm x 5 mm, about 5 mm x 10 mm, about 5 mm x 15 mm, about 5 mm x 20 mm, about 5 mm x 25 mm, about 5 mm x 30 mm, about 5 mm x 35 mm, about 5 mm x 40 mm, about 5 mm x 45 mm, about 5 mm x 50 mm, about 10 mm x 10 mm, about 10 mm x 15 mm, about 10 mm x 20 mm, about 10 mm x 25 mm, about 10 mm x 30 mm, about 10 mm x 35 mm, about 10 mm x 40 mm, about 10 mm x 45 mm, about 10 mm x 50 mm, about 15 mm x 15 mm, about 15 mm x 20 mm, about 15 mm x 25 mm, about 15 mm x 30 mm, about 15 mm x 35 mm, about 15 mm x 40 mm, about 15 mm x 45 mm, about 15 mm x 50 mm, about 20 mm x 20 mm, about 20 mm x 25 mm, about 20 mm x 30 mm, about 20 mm x 35 mm, about 20 mm x 40 mm, about 20 mm x 45 mm, about 20 mm x 50 mm, about 25 mm by 25 mm, about 25 mm by 30 mm, about 25 mm by 35 mm, about 25 mm by 40 mm, about 25 mm by 45 mm, about 25 mm by 50 mm, about 30 mm x 30 mm, about 30 mm

x 35 mm, about 30 mm x 40 mm, about 30 mm x 45 mm, about 30 mm x 50 mm, about 35 mm x 35 mm, about 35 mm x 40 mm, about 35 mm x 45 mm, about 35 mm x 50 mm, about 40 mm x 40 mm, about 40 mm x 45 mm, about 40 mm x 50 mm, 45 mm x 45 mm, 45 mm x 50 mm, or about 50 mm x 50 mm.

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In some embodiments, the grid bars are about 1 mm thick and/or regularly spaced to form an aperture/opening between about 5 mm x 5 mm to about 10 mm x 10 mm, and/or have a ratio of the grid bar area to the total grid area of about 15% to about 25%, thereby allowing the grids to exert even compression on the membranes to straighten them, thus shaping the cell spheroids into a membrane tissue.

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Membranes

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In various embodiments, the membrane holding device may further comprise two or more membranes/mesh layers configured to be disposed between the first and second plates, when the plates are coupled together. As used herein, the term “membrane”, “mesh”, “mesh layer” are understood to describe the same component and may therefore be used interchangeably.

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In various embodiments, the membranes are configured to be part of the tissue membrane construct. In various embodiments, the membranes are substantially planar. In various embodiments, the membranes are configured to be substantially flexible. This may facilitate implantation of the tissue membrane, as the tissue membrane may be sufficiently flexible to conform to different types of surfaces (e.g., flat surfaces, contoured surfaces) at an implantation site. In various embodiments, the membranes are configured to provide mechanical strength to a tissue membrane construct. In various embodiments, the membranes are configured to provide mechanical support to a cell layer, e.g., cell spheroid layer. In various embodiments, the membranes are configured to allow fluid e.g., culture medium to pass therethrough. In various embodiments

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therefore, the membranes are porous to fluid, e.g., air, liquid. In various embodiments, the membranes may comprise a plurality of pores.

In various embodiments, the membrane holding device may be configured to hold 2, 3, 4, 5, 6, 7 or 8 membranes. In various embodiments, the membranes are stackable. In various embodiments, the membranes may be arranged in a stacked manner when held between the first and second plates. In various embodiments, two immediately adjacent membranes may be configured to hold/sandwich a cell spheroid layer therebetween, when the membranes are stacked together.

In various embodiments, the membrane holding device may be configured to hold two membranes. For example, the membrane holding device may comprise, a first membrane with one side configured to contact the first plate and an opposite side configured to interface with a layer of cell spheroids; and a second membrane with one side configured to contact the second plate and an opposite side configured to interface with the layer of cell spheroids.

In various embodiments, the membranes are not particularly limited in terms of its shape and size and may be customized to fit its intended application. In various embodiments, the membranes may be fabricated using suitable techniques that are capable of producing pores with a desired pore size. In various embodiments, techniques for fabricating the membranes include, but are not limited to, weaving, knitting, braiding, 3D printing, ion track etching, freeze drying, lithography, electrospinning, phase inversion, solvent casting and particulate leaching. In various embodiments, the membranes may be obtained from commercially available sources/off-the shelf sources. For example, the membranes may be formed from a network of threads/fibers.

In various embodiments, the membranes may take the form of meshes formed using threads/membrane threads having a defined diameter. In various embodiments, the membrane thread may have a diameter of from about 1 μm to

about 200 μm . In various embodiments, the diameter of the membrane thread may fall in the range with start and end points selected from the following group of numbers: 1, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, and 200 μm .

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In various embodiments, the diameter of the membrane thread may be less than about 10 μm , less than about 20 μm , less than about 30 μm , less than about 40 μm , less than about 50 μm , less than about 60 μm , less than about 70 μm , less than about 80 μm , less than about 90 μm , less than about 100 μm , less than about 110 μm , less than about 120 μm , less than about 130 μm , less than about 140 μm , less than about 150 μm , less than about 160 μm , less than about 170 μm , less than about 180 μm , less than about 190 μm , or less than about 200 μm . In some embodiments, the diameter of the membrane thread is less than about 100 μm .

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In various embodiments, the plurality of pores in the membranes/mesh layers may have a pore size falling in the range of from about 0.1 μm (or 100 nm) to about 500 μm . In various embodiments, the pore size of the membranes may fall in the range with start and end points selected from the following group of numbers: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, and 500 μm . In some embodiments, the pore size of the membranes falls in the range of from about 150 μm to about 200 μm . In various embodiments, the pore size of the membrane is less than the average diameter of a cell spheroid.

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In various embodiments, the membranes may be made of a bioinert material. In various embodiments, the use of a bioinert material may advantageously discourage interaction of cells with the membranes, e.g., discourage cells from dissociating from the cell spheroid layer and propagating on the membranes instead. In various embodiments, the membrane of bioinert

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material may be made of a natural material, a synthetic material, a composite material, or a combination thereof. In various embodiments, the membrane of bioinert material may be made of a metal, a biomaterial or a polymer material. In various embodiments, the bioinert material may include, but is not limited to, 5 nylon (such as nylon 6), polymers (such as polycaprolactone (PCL), polyethylene (PE)), metals (such as titanium, stainless steel), natural silk, and the like.

In various embodiments, the membranes may be made of a biocompatible/bioactive material. In various embodiments, the 10 biocompatible/bioactive material may be a material that is coated with bioactive components and/or expresses bioactive ingredients from the membrane core. In various embodiments, the membrane of biocompatible/bioactive material may include, but is not limited to, a collagen membrane, a gelatin membrane, a decellularized tissue membrane (such as from the skin, bone, cartilage, 15 intestine), and the like. In various embodiments, the membrane of biocompatible/bioactive material may include a bioinert and/or biocompatible membrane that are coated with and/or contain bioactive compounds and/or drugs that induce biological responses from cells (such as inducing differentiation).

20 In various embodiments, the choice of using bioinert and/or biocompatible/bioactive membranes may depend on the specific requirements of an application, e.g., type of tissue to be engineered. For example, bioinert membranes may be used for an application to produce cartilage tissue. In various embodiments, the bioinert membranes may provide mechanical strength to the tissue membrane construct. In various embodiments, the biocompatible/bioactive 25 membranes may be in direct contact with the cells in the cell spheroid layer. For example, two layers of biocompatible membranes may be provided to hold/sandwich a cell spheroid layer therebetween, such that the cell spheroids are in direct contact with the biocompatible membranes. The two layers of 30 biocompatible membranes may be further sandwiched by two layers of bioinert membranes to provide mechanical support. In various embodiments, the membrane holding device may be configured to hold two bioinert membranes,

three bioinert membranes, four bioinert membranes, five bioinert membranes, six
bioinert membranes, seven bioinert membranes, eight bioinert membranes, two
biocompatible/bioactive membranes, three biocompatible/bioactive membranes,
four biocompatible/bioactive membranes, five biocompatible/bioactive
5 membranes, six biocompatible/bioactive membranes, seven
biocompatible/bioactive membranes, eight biocompatible/bioactive membranes,
two biocompatible/bioactive membranes and two bioinert membranes, three
biocompatible/bioactive membranes and three bioinert membranes, four
biocompatible/bioactive membranes and four bioinert membranes, and the like.

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Guide members

In various embodiments, the membrane holding device may further
comprise one or more guide/alignment member for facilitating alignment of the
15 first and second plates during coupling. In various embodiments, the guide
member ensures that the first and second plates are orientated correctly during
coupling.

In various embodiments, the guide member may take the form of a guide
20 pin disposed on the first plate, e.g., a protrusion with a specific cross-sectional
shape formed on the interior surface of the first plate, and the second plate
comprises an opening e.g., hole having the same shape as the cross section of
the guide pin. When orientated correctly, the opening in the second plate is
configured to receive the protrusion on the first plate, such that the first and
25 second plates are aligned when coupled to each other.

Coupling members

In various embodiments, the membrane holding device may further
30 comprise one or more coupling members configured for facilitating coupling of
the first plate to the second plate.

In various embodiments, the coupling members facilitate assembly and disassembly of the first and second plates. In various embodiments, the coupling members may facilitate the tightness of coupling of the first and second plates. In other words, the coupling members may be configured to allow adjustable control
5 of the compression applied on the tissue membrane construct. In various embodiments, the tightening of the first plate and the second plate of the membrane holding device presses the tissue membrane construct held therebetween firmly and evenly.

10 In various embodiments, the coupling members may comprise screws. For example, the first and second plates may comprise screw holes for allowing the screws to be applied. The first plate may comprise a first set of screw holes and the second plate may comprise a second set of screw holes in positions corresponding to the first set of screw holes. The screw holes may be
15 positioned/spaced at regular intervals on the first and second plates to ensure a substantially even distribution of compressive forces when the plates are coupled.

In various embodiments, the coupling members may comprise
20 clamps/clips, e.g., quick release or adjustable clamps. For example, clamps may be applied to couple the first and second plates. In various embodiments, the coupling members may include, but is not limited to, a magnetic latching mechanism, a hook and loop latching mechanism (such as Velcro®), a bioadhesive, and the like.

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Tissue membrane culture system and method of culturing

In various embodiments, there is provided a tissue membrane culture system comprising, a membrane holding device as disclosed herein, two or more
30 membranes as disclosed herein, said two or more membranes configured to be stacked and held in position between a first plate and a second plate of the membrane holding device, when the first and second plates are coupled to each

other; and a cell spheroid layer configured to be disposed between two adjacent membranes of the two or more membranes. In various embodiments, the tissue membrane culture system may further comprise a culture vehicle. In various embodiments, the culture vehicle may be a static culture vehicle such as plates, dishes, flasks or wells. In various embodiments, the culture vehicle may be a dynamic culture vehicle such as bioreactors, e.g., stirred bioreactors, perfusion bioreactors.

In various embodiments, there is provided a method of culturing/growing a cell spheroid comprising, providing a membrane holding device as disclosed herein; placing a first membrane on an interior surface of a first plate, placing a second plate over the first membrane such that the first and second plates are in coupling position, seeding cell spheroids to form a spheroid layer on the first membrane via one or more holes provided in the second plate, removing the second plate, placing a second membrane over the spheroid layer, placing the second plate over the second membrane, and coupling the first and second plates to each other.

Tissue membrane construct, tissue membrane, and uses thereof

In various embodiments, there is provided a tissue membrane construct comprising two or more membranes/mesh layers, each membrane comprising a plurality of pores; and a layer of cell spheroids disposed between at least one adjacent pair of the two or more membranes, e.g., any two adjacent membranes. In various embodiments, the tissue membrane construct may comprise a first membrane/mesh layer comprising a plurality of pores; a second membrane/mesh layer comprising a plurality of pores; and a layer of cell spheroids disposed between the first and second membranes.

In various embodiments, the tissue membrane construct may be assembled using the membrane holding device as disclosed herein. In various embodiments, cell spheroids may be assembled into a thin layer of tissue

membrane. In various embodiments, the two layers of mesh/membrane provide mechanical support without compromising on medium diffusion. In various embodiments, the mesh/membrane is substantially flexible and has optimal tensile strength for the final tissue membrane to be handled in orthopedic applications. In various embodiments, the cell for the cell spheroids may be an embryonic stem cell, an induced pluripotent stem cell, a mesenchymal stem cell, a primary cell (such as a chondrocyte, a fibroblast), a genetically modified cell, and the like.

10 In various embodiments, the tissue membrane construct may be stackable with one or more additional tissue membrane constructs to form a thicker tissue construct. In various embodiments, stacking of the tissue membrane constructs may be performed with or without the membrane holding device. For example, after harvesting tissue membrane constructs from the membrane holding devices
15 as disclosed herein, multiple tissue membrane constructs may be stacked together and immediately used for implantation if a thicker tissue is needed.

In various embodiments, the tissue membrane construct may be substantially devoid of a gel. In various embodiments, the membranes/mesh layers may be substantially devoid of a gel. In various embodiments, the layer of cell spheroids may be substantially devoid of a gel. The inventors have recognized that when the cell spheroids are suspended or embedded in gel (e.g., hydrogel, agarose gel), cell-to-cell contact, which is important for uniform and functional tissue development and maturation, may be reduced or impeded. In
20 various embodiments, enhanced cell-to-cell contact between neighbouring spheroids in the cell spheroid layer may advantageously promote uniform and functional tissue development and maturation.

In various embodiments, the tissue membrane construct may be kept
30 under suitable culture conditions to facilitate its development into a tissue membrane. In various embodiments, there is provided a tissue membrane comprising two or more membranes/mesh layers, each membrane comprising a

plurality of pores; and a layer of tissue disposed between at least one pair of the two or more membranes, e.g., any two adjacent membranes; wherein the layer of tissue is formed by fusion of a plurality of cell spheroids. In various embodiments, the tissue membrane construct may comprise a first
5 membrane/mesh layer comprising a plurality of pores; a second membrane/mesh layer comprising a plurality of pores; and a layer of tissue disposed between the first and second membranes; wherein the layer of tissue is formed by fusion of a plurality of cell spheroids.

10 In various embodiments, the dimensions (e.g., size, shape, and thickness) of the tissue membrane are tunable. For example, the dimensions of the tissue membrane may be tuned by adjusting the dimensions of the membrane holding device, membranes, cell spheroid seeding locations across the holes of the membrane holding device, and/or cell spheroid seeding densities.

15 In various embodiments, fusion of cell spheroids occurs when the plurality of cell spheroids in the cell spheroid layer combine to form a larger cohesive tissue structure. In various embodiments, fusion of cell spheroids may comprise three stages: an initial contact stage, a cell migration and rearrangement stage,
20 and a tissue maturation stage. In various embodiments, the initial contact stage occurs when multiple cell spheroids are brought into proximity (for example, deposited as a layer of cell spheroids between membranes) such that the outermost cells of the cell spheroids begin to adhere to one another. In various embodiments, the cell migration and rearrangement stage occur when cells in the
25 spheroids migrate toward each other and reorganize their cytoskeletons, facilitating fusion. Adhesion molecules, such as cadherins, may play an important role in this process. In various embodiments, the tissue maturation stage occurs when the boundaries between individual spheroids disappear over time, and the cells within the spheroids rearrange to form a continuous, larger tissue-like
30 structure. This stage is often aided by the extracellular matrix (ECM) proteins secreted by the cells and by factors such as growth factors added in the culture medium or incorporated in the membrane / mesh for release during culture.

In various embodiments, the tissue of the tissue membrane may include, but is not limited to, a skin tissue, a muscle tissue, a cartilage tissue, a vascular tissue, a bone tissue, a liver tissue, an intestinal / gut tissue, a lymphoid tissue, a pancreas tissue, a kidney tissue, a heart tissue, a neural tissue, and the like. In some embodiments, the tissue membrane is a cartilage tissue membrane. In some embodiments, the cartilage tissue membrane may be matured into e.g., a hypertrophic cartilage, a hyaline cartilage, and the like.

In various embodiments, the tissue membrane may be used as a live tissue and/or processed as a devitalized or decellularized tissue. Without wishing to be bound by theory, the tissue membranes generated by the present disclosure may be used for research (such as tissue models for drug testing) or therapeutic treating (such as implants). For example, hypertrophic cartilage tissue membrane may be used for bone fracture healing. For example, hyaline cartilage tissue membrane may be used to treat cartilage defects. In some embodiments, the tissue may be devitalized by methods known in the art such as boiling, freeze-thawing, freeze drying, and the like. In some embodiments, the tissue may be decellularized by methods known in the art e.g., using chemical methods (such as sodium dodecyl sulfate (SDS), Triton X-100, Sodium deoxycholate (SD), CHAPS), physical methods (such as supercritical CO₂, high hydrostatic pressure (HHP)), enzymatic methods (such as trypsin, deoxyribonuclease (DNase), ribonuclease (RNase)), and the like.

In various embodiments, the tissue membrane produced by the membrane holding device as disclosed herein may be used to produce cell-derived materials. In various embodiments, cell-derived matrix materials include, but are not limited to, collagen type 1, collagen type 2, collagen type 10, glycosaminoglycan, fibronectin, integrin, laminin, and the like. In various embodiments, the cell derived material may be extracted (such as using acid, enzyme extraction) and formulated into a composition, a scaffold such as a sponge, a hydrogel, an aerogel, a substrate, a fiber, a film, a patch, and the like.

In various embodiments, there is provided a tissue membrane or composition as disclosed herein, for use in therapy/medicine. In various embodiments, there is provided a tissue membrane or composition as disclosed
5 herein, for use in preventing and/or treating a disease. In various embodiments, there is provided use of a tissue membrane or composition as disclosed herein in the manufacture of a medicament for preventing and/or treating a disease. In various embodiments, there is provided a method of preventing and/or treating a disease in a subject in need thereof, the method comprises administering a tissue
10 membrane or composition as disclosed herein to the subject. In various embodiments, there is provided a method of enhancing tissue regeneration in a subject in need thereof, the method comprises administering a tissue membrane or composition as disclosed herein to the subject. In various embodiments, there is provided a method of preventing and/or reducing the severity of symptoms
15 caused by a disease in a subject in need thereof, the method comprises administering a tissue membrane or composition as disclosed herein to the subject. In various embodiments, the disease may include, but is not limited to, cartilage defects (such as osteoarthritis), bone defects, tendon defect and/or injury, ligament defect and/or injury, muscle defect and/or injury, and the like. In
20 various embodiments, hyaline cartilage generating using the tissue membrane may be used for implantation in cartilage defects such as osteoarthritis. In various embodiments, hypertrophic cartilage generated using the tissue membrane may be used for implantation in bone defects to enhance bone regeneration, tendon regeneration and/or ligament regeneration. For example, the hypertrophic
25 cartilage membrane may be used to wrap around a bone fracture to enhance fracture healing due to the presence of hypertrophic cartilage and the stabilizing effect of the meshes.

Method of producing a tissue membrane

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In various embodiments, there is provided a method of producing a tissue membrane, the method comprising, providing a membrane holding device

comprising a first part e.g., first plate and a second part e.g., second plate configured to be detachably coupled to the first plate; assembling a tissue membrane construct comprising a layer of cell spheroids disposed between a first membrane/mesh layer and a second membrane/mesh layer, such that the tissue
5 membrane construct is held in position between the first and second plates of the membrane holding device; and maintaining/keeping the membrane holding device with the tissue membrane construct under culture conditions to produce the tissue membrane.

10 In various embodiments, the method may be used to produce tissue membrane for any tissue that uses cell spheroids as building blocks. In various embodiments, the method may be used to scale up tissue manufacturing. In various embodiments, the method may be used to produce cell-derived materials. In various embodiments, the use of the membrane holding device may
15 advantageously apply a substantially even distribution of force e.g. compressive force, thereby facilitating the fabrication of a thin cell spheroid layer on the membranes.

In various embodiments, the membranes form part of the final tissue
20 membrane. In various embodiments, the tissue membrane construct is not limited to two membranes. In various embodiments, the tissue membrane construct may be assembled using two or more membranes arranged in a stacked manner. In various embodiments, a layer of cell spheroids may be provided between at least one adjacent pair of the two or more membranes, e.g., any two membranes
25 arranged/stacked adjacent to each other.

In various embodiments, the method may further comprise preparing the cell spheroids for use in assembling the tissue membrane construct. In various embodiments, preparing the cell spheroids may comprise expanding a cell
30 population and generating cell spheroids from the expanded cell population.

In various embodiments, cell spheroids may be formed from cells including, but not limited to, an embryonic stem cell, an induced pluripotent stem cell, a mesenchymal stem cell (MSC), a primary cell (such as a chondrocyte, a fibroblast, an osteoblast, and the like), a progenitor cell (such as a satellite cell, a periosteum cell, a perichondrium cell, and the like), and the like. In various
5 embodiments, the mesenchymal stem cell may be derived from e.g., multipotent stem cell, bone-marrow derived stem cell, adipose derived stem cell, and the like. In various embodiments, the chondrocyte may include an articular chondrocyte, an auricular chondrocyte, a costal chondrocyte, or a nasoseptal chondrocyte, and
10 the like. In various embodiments, the cell may be a genetically modified cell. In various embodiments, genetic modification of cells may include gene overexpression (such as genes for cartilage production, cell immortalization), and the like.

15 In various embodiments, a cell population may be expanded using a suitable expansion method known in the art, such as using two-dimensional (2D) culture (e.g., adherent cell culture on surfaces of cell culture flasks or plates) or suspension culture with cells freely floating in a culture medium, depending on the requirements of the cells. In various embodiments, the cell expansion method
20 is chosen such that differentiation potential (e.g., stemness of stem cells), phenotypes (e.g., phenotypes of primary chondrocytes or cell lines) and other characteristics of the cells can be maintained after expansion. In various embodiments, quantitative and/or qualitative procedures e.g., assays or visual observation using a microscope may be performed to confirm the presence of the
25 characteristics of the cells, before, during and/or after cell expansion. For example, a chondro-pellet assay may be used to confirm the presence of chondrogenic capacity of an expanded cell population.

In various embodiments, cell spheroids may be generated using a suitable
30 method known in the art, such as using a microwell plate, a hanging drop assay, a gel embedding/hydrogel embedding assay, a magnetic levitation assay, a spinner culture/spinner flask, a microfluidic chip, a bioreactor, a pellet culture, a

bioprinting process, and the like. In some embodiments, microwell plates may be used to produce cell spheroids due to its ease of scaling up and ability to produce spheroids that are consistent in terms of e.g., size and number of cells per spheroid. In various embodiments, cell spheroids may be kept on microwell plates for 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days before transferring to the membrane holders. In some embodiments, cell spheroids may be kept on microwell plates for 2 days before transferring to the membrane holders.

In various embodiments, the method may further comprise a step of seeding cell spheroids to form the layer of cell spheroids between the two membranes arranged/stacked adjacent to each other. In various embodiments, the cell spheroids may be seeded manually. In various embodiments, the cell spheroids may be seeded using automated seeding devices or liquid handling systems. In various embodiments, the cell spheroids may be seeded using a single channel or a multi-channel pipette. In various embodiments, wide-bore pipette tips may be used to handle the cell spheroids to avoid damaging the 3D structures of the cell spheroids. In various embodiments, the cell spheroids may be aspirated and dispensed at relatively low flow speeds during seeding to avoid disrupting the cell spheroids' structure.

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In various embodiments, cell spheroids may be seeded by pipetting a suspension of cell spheroids (e.g., cell spheroids suspended in culture medium) onto a membrane via one or more holes/chambers provided in the plates (e.g., second (top) plate) of the membrane holding device. In various embodiments, the cell spheroids settle down evenly on the membrane as medium flows through the membrane. In various embodiments, the design of the membrane holding device having a plurality of holes on the plates (e.g., holes/chambers defined by grids) may advantageously allow seeding of the cell spheroids into one or more holes e.g., chambers and at different spheroid densities across the grids. In various embodiments, the number of chambers filled with spheroids and their location may determine the size and shape of the tissue membrane. In various embodiments, the spheroid density in each chamber may determine the

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thickness of the tissue membrane. Therefore, this allows control of the size, shape, and thickness of the resulting tissue membrane.

In various embodiments, the seeding step may comprise, (i) placing the first membrane on the first (bottom) plate; (ii) placing the second (top) plate relative to the first plate such that the first membrane is disposed between the first and second plates; (iii) depositing cell spheroids onto the first membrane via a plurality of holes provided in the second plate to form the layer of cell spheroids; (iv) removing the second plate; (v) positioning the second membrane over the layer of cell spheroids such that the layer of cell spheroids is disposed between the first and second membranes; and (vi) coupling the first and second plates to each other. The above steps, e.g., steps (ii) to (v), may be repeated to assemble tissue membrane constructs with more than two membranes. For example, after step (v), step (ii) may be repeated by placing the second (top) plate relative to the first plate such that the first and second membranes are disposed between the first and second plates. Step (iii) may be repeated by depositing cell spheroids onto the second membrane via a plurality of holes provided in the second plate to form a second layer of cell spheroids. Step (iv) may be repeated by removing the second plate. Step (v) may be repeated by positioning a third membrane over the second layer of cell spheroids such that the second layer of cell spheroids is disposed between the second and third membranes. Thereafter, step (vi) is performed by coupling the first and second plates to each other.

In various embodiments, the method may be devoid of a step of using gels when assembling the tissue membrane construct. For example, the step of seeding cell spheroids to form the layer of cell spheroids may be devoid of using a gel to suspend the cell spheroids. The inventors have recognized that when the cell spheroids are suspended or embedded in gel (e.g., hydrogel, agarose gel), cell-to-cell contact, which is important for uniform and functional tissue development and maturation, may be reduced or impeded. In various embodiments, the cell spheroid layer is deposited without using a gel, which may

promote cell-to-cell contact between neighbouring spheroids, and in turn, promote uniform and functional tissue development and maturation.

In various embodiments, the membranes comprise a plurality of pores, and the plurality of pores have an average pore size which is smaller than an average diameter of the cell spheroids. In various embodiments therefore, the first and second membranes are capable of containing the cell spheroids therebetween (e.g., by preventing the cells spheroids from falling through the pores of the membranes) but also allow medium perfusion and/or diffusion across the plurality of pores. In some embodiments, the plurality of pores of the membranes may have an average diameter falling in the range of from about 150 μm to about 200 μm . In some embodiments, the cell spheroids may have an average diameter falling in the range of from about 200 μm to about 300 μm . Without wishing to be bound by theory, the membrane's pore size range of about 150 to about 200 μm is smaller than the spheroids' diameter (about 200 μm to about 300 μm) so that it can contain the spheroids but also allows medium perfusion and/or diffusion. The mesh thread of 100 μm or less allows the bulk of the final tissue membrane to come from the tissue (such as cartilage tissue) and not the mesh.

In various embodiments, the cell spheroids may have an average diameter falling in the range of from about 50 μm to about 2000 μm . In various embodiments, the cell spheroids may have an average diameter falling in the range with start and end points selected from the following group of numbers: 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550, 1600, 1650, 1700, 1750, 1800, 1850, 1900, 1950, and 2000 μm .

In some embodiments, the cell spheroids may have an average diameter falling in the range of from about 200 μm to about 300 μm . Without wishing to be bound by theory, the inventors have found that cell spheroids of 200 to 300 μm may be the optimum building blocks for generating large complex tissues *in vitro*. For example, spheroids are important for cartilage tissue generation as the

maturation of cartilage tissue depends on the condensation process during spheroid formation. However, current methods in the art have difficulties such as the spheroids fusing together into a thick tissue that hinders medium diffusion and causes necrosis; having structures that are too weak for orthopedic applications; or requiring expensive equipment with low throughput. Various embodiments of the present disclosure address these problems with cell spheroids that are assembled into a thin layer of tissue membrane. In various embodiments, the two layers of mesh / membrane sandwiching the cell spheroid layer provide mechanical support without compromising on medium diffusion. In various embodiments, the mesh / membrane is substantially flexible and has optimal tensile strength for the final tissue membrane to be handled in orthopedic applications.

In various embodiments, the cell spheroids which are suspended in medium are pipetted into the chambers and settle down evenly on the mesh as medium flows through the mesh. The design of the membrane holding device allows seeding of the spheroids into one or more chambers and at different spheroid densities across the grids. The number of chambers filled with spheroids and their location determines the size and shape of the tissue membrane. The spheroid density in each chamber determines the thickness of the tissue membrane. Therefore, this allows control of the size, shape, and thickness of the resulting tissue membrane.

In various embodiments, the cell spheroids may comprise from about 100 cells to about 500,000 cells per spheroid. In various embodiments, the cell spheroids may comprise about 100, about 500, about 1000, about 1500, about 2000, about 2500, about 3000, about 3500, about 4000, about 4500, about 5000, about 5500, about 6000, about 6500, about 7000, about 7500, about 8000, about 8500, about 9000, about 9500, about 10000, about 15000, about 20000, about 25000, about 30000, about 35000, about 40000, about 45000, about 50000, about 55000, about 60000, about 65000, about 70000, about 75000, about 80000, about 85000, about 90000, about 95000, about 100000, about 150000,

about 200000, about 250000, about 300000, about 350000, about 400000, about 450000, or about 500000 cells per spheroid. It is recognized that cell sizes may vary across species and across different parts of a body. Therefore, the number of cells per spheroid may depend on the cell type. For example, it may be possible
5 to have more cells per spheroids for cell types with relatively smaller sizes. In some embodiments, the cell spheroid may comprise 5000 cells per spheroid. In some embodiments, cell spheroids having e.g., up to 250000 cells per spheroid may lead to a necrotic core. In some embodiments therefore, cell spheroids having e.g., up to 250000 cells per spheroid may be used as a disease model.

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In various embodiments, the cell spheroids may be seeded at a density falling in the range of from about 500 to about 5000 spheroids per cm^2 of a membrane, e.g., first (bottom) membrane. In various embodiments, the cell spheroids may be seeded at a density falling in the range with start and end points
15 selected from the following group of numbers: 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900, 4000, 4100, 4200, 4300, 4400, 4500, 4600, 4700, 4800, 4900, 5000, 5100, 5200, 5300, 5400, 5500, 5600, 5700, 5800, 5900, 6000, 6100, 6200,
20 6300, 6400, 6500, 6600, 6700, 6800, 6900, 7000, 7100, 7200, 7300, 7400, 7500, 7600, 7700, 7800, 7900, 8000, 8100, 8200, 8300, 8400, 8500, 8600, 8700, 8800, 8900, 9000, 9100, 9200, 9300, 9400, 9500, 9600, 9700, 9800, 9900 and 10000 cell spheroids per cm^2 . In some embodiments, higher seeding densities may be used for cell types with relatively smaller sizes. In some embodiments, the cell
25 spheroids may be seeded at a density falling in the range of from about 1000 to about 3000 spheroids per cm^2 .

In various embodiments, the method may further comprise fabricating the first and second membranes in the form of meshes. In various embodiments, the
30 meshes may be fabricated using threads. In various embodiments, the meshes may be fabricated using threads having an average diameter of less than about 200 μm .

In various embodiments, the tissue membrane (i.e., resulting tissue membrane formed from the tissue membrane construct) may have an average thickness falling in the range of from about 50 μm to about 2000 μm . In various
5 embodiments, the average thickness of the tissue membrane may fall in the range with start and end points selected from the following group of numbers: 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1550, 1600, 1650, 1700, 1750, 1800, 1850, 1900, 1950, and 2000 μm .

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In some embodiments, the resulting tissue membrane may have a thickness falling in the range of from about 200 μm to about 600 μm . Without wishing to be bound by theory, the first and second plates of the membrane holding device that presses firmly and evenly on the meshes exert sufficient and
15 even compression to straighten the meshes, thus constraining the cell spheroids into a thin tissue (about 200 μm to 600 μm)

In various embodiments, the membrane holding device may produce a tissue membrane having dimensions (width x length) of about 5 mm x 10 mm,
20 about 5 mm x 15 mm, about 5 mm x 20 mm, about 5 mm x 25 mm, about 5 mm x 30 mm, about 5 mm x 35 mm, about 5 mm x 40 mm, about 10 mm x 10 mm, about 10 mm x 15 mm, about 10 mm x 20 mm, about 10 mm x 25 mm, about 10 mm x 30 mm, about 10 mm x 35 mm, about 10 mm x 40 mm, about 15 mm x 15 mm, about 15 mm x 20 mm, about 15 mm x 25 mm, about 15 mm x 30 mm, about
25 15 mm x 35 mm, about 15 mm x 40 mm, about 20 mm x 20 mm, about 20 mm x 25 mm, about 20 mm x 30 mm, about 20 mm x 35 mm, about 20 mm x 40 mm, about 30 mm x 30 mm, about 30 mm x 35 mm, about 30 mm x 40 mm, about 35 mm x 35 mm, about 35 mm x 40 mm, or about 40 mm x 40 mm. In some embodiments, the membrane holding device may be a small membrane holder
30 that produces a tissue membrane of about 5 mm x 10 mm. In some embodiments, the membrane holding device may be a larger membrane holder that produces a tissue membrane of about 40 mm x 40 mm.

In various embodiments, the membrane holding device holding the tissue membrane construct may be maintained under suitable culture conditions to form the tissue membrane. In various embodiments, the membrane holding device
5 holding the tissue membrane construct may be maintained under static culture conditions (e.g., in well plates or dishes), dynamic culture conditions (e.g., bioreactor) or a combination thereof. For example, a membrane holding device holding the tissue membrane construct may be first cultured under static conditions to facilitate adhesion of the cell spheroids to one another. Thereafter,
10 the membrane holding device may be transferred to a bioreactor to be cultured under dynamic culture conditions. In various embodiments, culture conditions such as temperature, gas environment (e.g., oxygen concentration, carbon dioxide concentration), humidity, pH, composition of culture medium, presence of growth factors, monitoring, and feeding (e.g., by media changes) may depend on
15 the types of tissue membrane (e.g., cartilage, bone or muscle tissue membrane) to be produced. It will be appreciated that a person skilled in the art would be able to choose the appropriate culture conditions to produce the tissue membrane.

In various embodiments, the method may further comprise stacking
20 together a plurality of membrane holding devices to produce a plurality of tissue membranes. In various embodiments, multiple membrane holding devices may be stacked together to allow scale-up manufacturing of multiple tissue membranes in a larger vessel/bioreactor. In various embodiments, the method may be scaled up to produce cell-derived matrix material such as but is not limited
25 to collagen type 1, collagen type 2, collagen type 10, glycosaminoglycan, fibronectin, integrin, laminin, and the like. In various embodiments, the cell derived material can be extracted (such as using acid, enzyme extraction) and formulated into a composition, a scaffold such as a sponge, a hydrogel, an aerogel, a substrate, a fiber, a film, a patch, and the like.

BRIEF DESCRIPTION OF FIGURES

FIG. 1A to FIG. 1E are a series of schematic drawings illustrating a method of producing a tissue membrane, e.g. cartilage tissue membrane, from cell spheroids *in vitro* in an example embodiment.

FIG. 1A is a schematic drawing showing cell spheroids made in a microwell plate in the example embodiment.

FIG. 1B is a schematic drawing showing an assembly of a tissue membrane construct on a membrane holding device in the example embodiment.

FIG. 1C is a schematic drawing showing an *in vitro* culture of the assembled tissue membrane construct in the example embodiment.

FIG. 1D is a schematic drawing showing apparatus for processing the tissue membrane construct in the example embodiment.

FIG. 1E is a schematic drawing showing an application of the tissue membrane (such as cartilage tissue membrane) on a bone fracture site in the example embodiment.

FIG. 2 is a schematic drawing of a membrane holding device / membrane holder in an example embodiment.

FIG. 3 is a schematic drawing showing a grid comprising a network of grid bars in an example embodiment. The area demarcated by dashed line represents the total grid area. The area shaded in grey represents the area occupied by the grid bars (i.e., grid bar area). The ratio of the grid bar area over the total grid area is about 15-25%. Grid bars are about 1-mm thick and regularly spaced to form a plurality of apertures, each aperture having dimensions of between about 5×5 mm to about 10×10 mm.

FIG. 4A and FIG. 4B are photographs showing membrane holding devices / membrane holders of different dimensions.

5 FIG. 4A is a photograph showing a top view of a small membrane holding device in an example embodiment. The small membrane holder can make a tissue membrane having dimensions about 5×10 mm for *in vitro* or small animal experiments. Scale bar = 1 cm.

10 FIG. 4B is a photograph showing a top view of a large membrane holding device in an example embodiment. The large membrane holder can make a tissue membrane having dimensions of up to about 40×40 mm for a large animal experiment or clinical study. Scale bar = 1 cm.

15 FIG. 5A to FIG. 5D are a series of schematic drawings illustrating a method of assembling cell spheroids on a membrane holder in an example embodiment.

FIG. 5A is a schematic drawing showing a first part (bottom part) of the membrane holder in the example embodiment. A first mesh (e.g., 1-layer mesh)
20 is placed over a first grid disposed at the bottom part of the membrane holder.

FIG. 5B is a schematic drawing showing a second part (top part) of the membrane holder placed over the bottom part of the membrane holder in the example embodiment. The top part of the membrane holder comprises a second
25 grid. The first and second grids are aligned when the top and bottom parts of the membrane holder are aligned. The top part of the membrane holder is put over the mesh. Cell spheroids are pipetted into chamber(s)/grid chamber(s) formed by the aligned holes of the first and second grids. The number of chambers to be filled with spheroids and their location determine the size and shape of the
30 resulting tissue membrane. The spheroid density in each chamber determines the thickness of the tissue membrane.

FIG. 5C is a schematic drawing showing the bottom part of the membrane holder in the example embodiment. After spheroid seeding, the top part of the membrane holder is removed in order to place another mesh (second mesh) over the spheroid layer measuring 20×20 mm.

5

FIG. 5D is a schematic drawing showing the top part of the membrane holder coupled to the bottom part of the membrane holder in the example embodiment. After the second mesh is placed over the spheroid layer such that the spheroid layer is sandwiched between the first and second meshes, the top part of the membrane holder is placed over the bottom part of the membrane holder and both parts are secured/tightened with screws.

10

FIG. 6A to FIG. 6C are microscopy images showing the development of a cartilage tissue membrane in culture in an example embodiment.

15

FIG. 6A is a bright-field microscopy image of the cartilage tissue membrane 3 days after cell spheroids seeding in an example embodiment. The spherical shape of individual cell spheroids was still recognizable from the microscopy image. Scale bar = 400 μm.

20

FIG. 6B is a fluorescence microscopy image of live cell staining (Calcein) of the cartilage tissue membrane after 1 week showing the fused tissue membrane layer in the example embodiment. Scale bar = 400 μm.

25

FIG. 6C is a photograph of the final cartilage tissue membrane with a 10×5×0.5mm (L×W×H) cartilage tissue sandwiched between two layers of Nylon mesh. Scale bar = 1 cm.

FIG. 7 is a bar graph showing a gene expression profile of a cartilage tissue membrane at day 14 of culture. Donor-derived hMSCs (Lonza) were expanded until passage 6 and formed into spheroids (5,000 cells/spheroid) on a microwell plate. 2000 spheroids were assembled into a 5×10 mm tissue

30

membrane supported by a 6-well format membrane holder and the tissue was cultured in chondrogenic medium for cartilage development. On day 14, the tissue membrane was harvested, and gene expression assays were performed using specific TaqMan probes for human SOX9, COL2A, ACAN, RUNX3, SP7, COL1A and COL10A. The expression level was compared to that of Day 0 (before starting chondrogenic medium) and displayed as fold change. Bar graph shows mean plus standard deviation (n=3).

FIG. 8 is a bar graph showing the results of a study optimizing cell number per spheroids. Spheroids of 5,000 or 25,000 cells were assembled on membrane holders and cultured for 14 days in chondrogenic medium. Gene expression results showed that the 5,000-cell group had significantly higher expression of cartilage matrix gene (COL2A, ACAN) and hypertrophic markers (RUNX2, SP7, COL1A and COL10A). Bar graph shows mean and standard deviation (n=3); multiple T-test with Bonferroni correction was performed; **: p<0.01, ***: p<0.001.

FIG. 9 is a bar graph showing the results of a study optimizing time to transfer cell spheroids to membrane assembly. Spheroids of 5,000 cells were assembled on the membrane holders after 1, 2 or 3 days in the microwells. Gene expression analysis was performed after 14 days. The results showed that the spheroids that were transferred after 1 day had the least expression of chondrogenic marker (SOX9) and cartilage matrix gene (COL2A, ACAN). Bar graph shows mean and standard deviation (n=3); multiple T-test with Bonferroni correction was performed; ***: p<0.001, ns: not significant.

FIG. 10 is a set of images showing histological analysis of a cartilage tissue membrane. Alcian-Blue-stained, longitudinal section of the cartilage tissue membrane showed the typical morphology of cartilage tissue with abundant extracellular matrix and large round cell lacunae. The mesh layers were embedded inside the cartilage tissue. The whole tissue membrane was approximately 500-600 µm thick. (*) denotes the area of terminally differentiated

hypertrophic chondrocytes. Scale bar of top left image = 5 mm. Scale bar of top right image = 100 μ m. Scale bar of bottom image = 1mm.

FIG. 11A to FIG. 11G are a series of images showing a process of cartilage
5 membrane production in an example embodiment.

FIG. 11A is a photograph showing an assembly of a tissue membrane
construct in the example embodiment. Scale bar = 5 mm.

10 FIG. 11B is a photograph of cartilage membranes harvested after *in vitro*
culture in the example embodiment.

FIG. 11C is a microscopy image of a longitudinal section of the cartilage
membrane stained with hematoxylin and eosin (H&E) in the example
15 embodiment. Scale bar = 100 μ m.

FIG. 11D is a microscopy image of a longitudinal section of the cartilage
membrane stained with Alcian blue in the example embodiment. Scale bar = 100
20 μ m.

FIG. 11E is a microscopy image of a longitudinal section of the tissue
membrane stained with collagen type 2 antibody in the example embodiment.
Scale bar = 100 μ m.

25 FIG. 11F is a pair of scanning electron microscope (SEM) images of the
tissue membrane in the example embodiment. Scale bar of overview image (left)
= 100 μ m. Scale bar of magnified image (right) = 10 μ m

FIG. 11G is a SEM image of a native porcine cartilage tissue for
30 comparison with FIG. 11F. Scale bar = 10 μ m.

FIG. 12A to FIG. 12D are images and graphs related to mechanical testing of a cartilage membrane in an example embodiment.

FIG. 12A is a photograph of a cartilage membrane that was punched out
5 at 5-mm diameter before a mechanical test in the example embodiment.

FIG. 12B is a photograph of a mechanical test set-up in the example embodiment.

10 FIG. 12C is a force-time graph showing equilibrium compression of the cartilage membranes during an unconfined compression test in the example embodiment.

FIG. 12D is a stress-strain graph showing stress-strain curves of two
15 samples generated from the stress relaxation during the unconfined compression test in the example embodiment.

FIG. 13A and FIG. 13B are microscopy images of a harvested cartilage membrane which was subcutaneously implanted in a rat in an example
20 embodiment.

FIG. 13A is a microscopy image of the cartilage tissue membrane stained with H&E in the example embodiment.

25 FIG. 13B is a microscopy image of the cartilage tissue membrane stained with Alcian blue in the example embodiment.

FIG. 14 is a series of microscopy images and photographs showing Nylon meshes seeded using different densities of cell suspensions in a comparative
30 example.

FIG. 15 is a graph showing gene expression profiles of samples taken from Nylon meshes seeded using different densities of cell suspensions in the comparative example.

5

DETAILED DESCRIPTION OF FIGURES

Example embodiments of the disclosure will be better understood and readily apparent to one of ordinary skill in the art from the following discussions and if applicable, in conjunction with the figures. It should be appreciated that other modifications related to structural, biological, chemical and material changes may be made without deviating from the scope of the invention. Example embodiments are not necessarily mutually exclusive as some may be combined with one or more embodiments to form new exemplary embodiments. The example embodiments should not be construed as limiting the scope of the disclosure.

The present disclosure discloses a method of producing a tissue membrane (such as cartilage tissue membrane) from cell spheroids which comprises the following steps of i) cell expansion and generation of cell spheroids ii) assembling of cell spheroids into a tissue membrane construct utilizing a membrane holding device and iii) maturation of the tissue membrane construct into the tissue membrane (such as cartilage tissue membrane) *in vitro* and post-processing. FIG. 1A to FIG. 1E summarize the tissue membrane (such as cartilage tissue membrane) production method according to an example embodiment.

In the example embodiment, cell spheroids (e.g., 100) are made using a microwell plate (see FIG. 1A). As shown in FIG. 1B, the cell spheroids (e.g., 100) harvested from the microwell plate are evenly spread as a cell spheroid layer (102) disposed between two membranes (such as first mesh (104) and second mesh (106)). The first mesh (104) and second mesh (106) sandwiching the cell

spheroid layer (102) form a tissue membrane construct (108) supported by a membrane holding device (110). The membrane holding device (110) comprises a first (bottom) part (112) and a second (top) part (114). This allows efficient medium diffusion while maintaining the spheroid structure for tissue (such as cartilage) development. The membrane holding device (110) loaded with the tissue membrane construct (108) is cultured in a suitable medium (such as chondrogenic medium) to induce tissue (such as cartilage) development to form a tissue membrane (see FIG. 1C). The tissue membrane is processed (e.g., maintained in a bioreactor culture such as a spinner flask under suitable conditions) depending on its applications into its final form (see FIG. 1D). The final tissue membrane (116) comprises tissue (such as cartilage tissue) sandwiched between two layers of membranes (such as first mesh (104) and second mesh (106)). The tissue membrane (116) (such as cartilage tissue membrane) can be utilized for orthopaedic applications such as bone/cartilage reconstruction (see FIG. 1E).

The present disclosure also discloses a membrane holding device to facilitate the making of the tissue membrane (such as cartilage tissue membrane).

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FIG. 2 is a schematic drawing of a membrane holding device / membrane holder (200) in an example embodiment. The membrane holding device (200) comprises a first part e.g., first plate (212) and a second part e.g., second plate (222). The first plate (212) comprises a first grid (214), said first grid (214) comprising a network of grid bars (e.g., 216) defining a plurality of holes (e.g., 218). The second plate (222) comprises a second grid (224), said second grid (224) comprising a network of grid bars (e.g., 226) defining a plurality of holes (e.g., 228). The first plate (212) and the second plate (222) are configured to be detachably coupled to each other. The first plate (212) and the second plate (222) are configured to exert a substantially even compression on a tissue membrane construct (208) held between the first plate (212) and second plate (222). The tissue membrane construct (208) comprises a layer of cell spheroids (also

30

referred to as cell spheroid layer) (202) disposed between a first membrane e.g., first mesh (204) and a second membrane e.g., second mesh (206).

5 In the example embodiment, the first grid (214) and second grid (224) are configured to be aligned with each other when the plates (212, 222) are coupled together. The aligning grids (214, 224) press firmly and evenly on the meshes (204, 206) to straighten the meshes (204, 206), thus constraining the spheroids in the cell spheroid layer (202) into a thin tissue layer. The grid design is optimized for efficient compression and medium perfusion. The second plate (222) is used
10 to facilitate seeding of the cell spheroids evenly during assembly. When coupled by tightening together, the grids (214, 224) form a plurality of chambers separated by the aligned networks of grid bars (e.g., 216, 226). Each hole (e.g., 218) in the first plate (212) is configured to be substantially aligned with a corresponding hole (e.g., 228) of the same shape and size in the second plate (222) to form a
15 chamber, thereby facilitating movement of substances e.g., flow of medium through both holes. Each chamber encloses a small part of the tissue membrane. This design allows users to easily control the size, shape and thickness of the tissue membrane.

20 In the example embodiment, the first and second plates (212, 222) are coupled to each other using a plurality of screws (e.g., 230). The first and second plates (212, 222) comprise screw holes (e.g., 232) for allowing the screws to be applied. The first plate (212) comprises a first set of screw holes (e.g., 232) and the second plate comprises a second set of screw holes (e.g., 232) in positions
25 corresponding to the first set of screw holes (e.g., 232). The screw holes (e.g., 232) are positioned/spaced at regular intervals on the first and second plates (212, 222) to ensure a substantially even distribution of compressive forces when the plates (212, 222) are coupled.

30 In the example embodiment, the membrane holding device (200) further comprise guide/alignment members (234, 236) for facilitating alignment of the first and second plates (212, 222) during coupling. The guide members (234, 236)

ensures that the first and second plates (212, 222) are orientated correctly during coupling. The guide members take the form of a pair of guide pins (e.g., 234) disposed on opposite ends of the first plate (212), e.g., a protrusion with a specific cross-sectional shape formed on an interior surface of the first plate (212). The
5 second plate (222) comprises a pair of openings, e.g., a pair of cut-outs (e.g., 236) having the same shape as the cross section of the guide pins (e.g., 234), disposed at opposite ends of the second plate (222). When orientated correctly, the cut-outs (e.g., 236) in the second plate (222) are configured to receive the guide pins (e.g., 234) on the first plate (212), such that the first and second plates
10 (212, 222) are aligned when coupled to each other.

EXAMPLES

15 Cell expansion and generation of cell spheroids

The method of producing a tissue membrane (such as a cartilage tissue membrane) of the present disclosure is applicable for any cell type that has previously been used in the art for tissue engineering (TE) (such as cartilage
20 tissue engineering). The cell types may include, but are not limited to, stem cells (such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPS), mesenchymal stem cells (MSCs, bone-marrow or adipose derived), primary cells (such as chondrocytes (articular, auricular, costal, nasoseptal), fibroblasts), and the like. The cell types may also be genetically modified to immortalize the cells
25 and/or overexpress certain genes that benefit tissue (such as cartilage) production (such as collagen type 2).

Cell expansion can be done using any suitable methods in the art (e.g., on 2D surface or in suspension). The method of cell expansion is chosen to maintain
30 the stemness (of stem cells) or maintain the cells' phenotypes (of primary chondrocytes or cell lines). In addition, one or more tests are performed to ensure

that the expanded cells are confirmed to have the full capacity (such as chondrogenic capacity) for making tissue matrix (such as cartilage matrix) *in vitro*.

To confirm the functional capacity (such as chondrogenic capacity) of the expanded cells, a standard functional assay (such as chondro-pellet assay) can be performed. In a chondro-pellet assay, 250,000 cells are spun down in a conical bottom tube in chondrogenic medium with growth factors such as transforming growth factor beta (TGF β) and dexamethasone. The cells should aggregate into a single pellet, which when cultured for a period of time (such as for 14-21 days) should mature into a cartilage tissue. This tissue should be characterized by increased expression of chondrogenic genes (e.g., SOX9, collagen type 2, aggrecan) and histological features of cartilage tissue (e.g., positively stained for Alcian Blue). Only cells that are successfully expanded and confirmed to have full functional capacity (such as chondrogenic capacity) are utilized to produce a tissue membrane (such as cartilage tissue membrane) using the method of the present disclosure.

Next, the expanded cells are made into cell spheroids of e.g., 5,000-10,000 cells/spheroid. This can be done using any suitable methods in the art such as hanging drops, microwells, spinner flask, magnetic levitation, or hydrogel embedding. A method that offers a high throughput and minimum interference to the cells should be chosen. For example, microwells may be used to make cell spheroids since this method is easy to scale up and is capable of producing consistent spheroids. Other methods such as hydrogel-embedded spheroids may be contaminated with hydrogel and its effect on chondrogenic culture should be tested in advance.

Assembling of cell spheroids into a tissue membrane utilizing a membrane holding device

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The inventors have recognized that small cell spheroids (e.g., having average diameters of about 200-300 μ m) may serve as optimum building blocks

for making larger complex tissue *in vitro*. For example, spheroids are important for cartilage tissue engineering because the maturation of cartilage tissue depends on the condensation process during spheroid formation.

5 However, the inventors have also recognized that there are several problems if cell spheroids are simply put together to make a larger cartilage tissue. For example, the spheroids tend to fuse together into a thick tissue, which hinders medium diffusion and causes necrosis core as well as growth factor gradients resulting in non-homogeneous development. Efforts in the art have
10 been made to physically separate the spheroids e.g., by embedding them in a hydrogel or by using a needle array. However, the methods in the art include limitations such as the hydrogel impeding medium diffusion, which causes its structure to be too weak for orthopaedic applications. In addition, the needle arrays in the art requires expensive equipment, and the throughput is low.

15 To address the limitations in the art, the present disclosure provides a method in which the cell spheroids are assembled into a thin layer (such as 200-600 μm thick) depending on the spheroid density. This spheroid layer is sandwiched between two layers of membranes (such as meshes) which provide
20 excellent mechanical support without compromising on medium diffusion.

Embodiments of the mesh as disclosed herein may include the following features:

25 The mesh may be made of a bioinert material. The bioinert material is part of the final tissue membrane and should not interact well with the cells, as this may cause cells to dissociate from the spheroids and spread on the mesh instead. For example, the mesh may be made of a synthetic polymer (such as Nylon 6) which is commonly used in orthopaedics. Meshes made of other bioinert
30 materials may be used as well, such as but is not limited to, other polymers (e.g., PCL, PE), certain metals (e.g., titanium, stainless steel), natural silk, and the like.

The mesh should have a pore size range (such as 150-200 μm) smaller than the average diameter of the cell spheroids (such as 200-300 μm) so that the mesh can contain the spheroids but allows efficient medium perfusion.

- 5 The thread for forming the mesh should be thin (such as a diameter of less than 100 μm) so that the bulk of the final tissue membrane come from the tissue (such as cartilage tissue), and not from the mesh material.

- 10 The mesh should be substantially flexible and possess sufficient tensile strength so that the final tissue membrane can be handled in various applications (such as orthopaedic applications). For example, the mesh should be capable of being wrapped around tissue defects (such as bone and cartilage defects) and capable of being pulled and tightened using surgical suture.

- 15 To support the making of the tissue membrane (such as cartilage tissue membrane) using the method of the present disclosure, a membrane holding device is developed to exert a substantially even compression on the meshes to constrain the cell spheroids into a relatively thin layer (such as having a thickness of about 200-600 μm).

20

Embodiments of the membrane holding device as disclosed herein may include the following features:

- 25 The membrane holding device comprises two parts (top and bottom) with grids that align to each other. The grid surface in contact with the meshes is smooth and flat. The ratio of area occupied by the network of grid bars to total area of the grid is about 15% to 25% (see FIG. 3). This ratio is optimized for applying a substantially even compression on the meshes and maximizing medium perfusion.

30

The grid bars are 1-mm thick and regularly spaced to form apertures/openings each having dimensions of between about 5×5 mm to about

10×10 mm (see FIG. 3). This design ensures a substantially even compression to keep the meshes flat and prevent mesh bulging during cell spheroid fusion.

5 If the aperture is too large, the meshes tend to bulge during spheroid fusion, resulting in the formation of a thick and uneven tissue membrane. If the aperture is too small, the actual tissue area is reduced; medium perfusion is compromised; the grid tends to trap bubbles more easily, and imaging of tissue membrane is more difficult. The grid bar/total grid area is optimized by testing and calculated based on these parameters.

10

The top part of the membrane holding device is used to seed the cell spheroids evenly during assembly (see FIG. 5A to FIG. 5D). The grids and the mesh form multiple chambers separated by the grid bars. The spheroids, suspended in medium, are pipetted into these chambers (see FIG. 5B). As
15 medium flows through the mesh, the spheroids settle down evenly on the mesh.

The membrane holding device facilitates ease of tuning the size, shape and thickness of the tissue membrane, by seeding the spheroids into one, two or multiple chambers, and at different cell spheroid density across the grid. A density
20 of about 1,000 to 3,000 spheroids per cm² (with about 5,000 cells per spheroids) may result in a tissue membrane thickness between about 200 to 600 μm.

By using the device for seeding, the spheroids automatically avoid areas where the grids press on each other, thus avoiding being pressed directly by the
25 grids (see FIG. 5C).

During assembly, the two parts of the membrane holding device are tightened together by screws which are spaced regularly to ensure substantially even and good compression on the mesh (see FIG. 5D).

30

The device can be scaled to accommodate the tissue membrane's size. For example, a smaller membrane holder e.g., in a 6-well plate format may be

fabricated using techniques such as 3D printing, which can produce tissue membranes having dimensions of e.g., 5×10 mm for *in vitro* or small animal experiment (see FIG. 4A). In another example, a larger membrane holder e.g., in a 10-cm petri dish format may be fabricated using techniques such as 3D printing, which can produce tissue membranes having dimensions of e.g., up to 40×40 mm which is clinically relevant (see FIG. 4B).

The membrane holding device may be made using any hard and bioinert material such as metal (e.g., stainless steel) or hard plastic (e.g., PLA, PEEK), using manufacturing techniques such as machining or 3D printing.

Maturation of the cartilage tissue membrane *in vitro* and post-processing

Once the membrane holder secures the tissue membrane inside, the membrane holder can be handled easily for medium change, imaging and performing any standard *in vitro* assays.

For medium change, the membrane holder (with tissue membrane inside) can be lifted and placed into a new well plate or petri dish with new medium.

For scale-up, multiple membrane holders can be cultured in a spinner flask or bioreactor with a large amount of medium. The medium may include for example chondrogenic medium for cartilage differentiation/maturation. The exact formulation depends on the cell types and stage of tissue (such as cartilage) development. The protocol should be optimized by the users and tested in advance using a standard assay (such as chondro-pellet assay). Once tested and confirmed, the medium can be used directly for the tissue membrane (such as cartilage tissue membrane) culture without any modification.

For imaging, the tissue membrane can be observed easily using any standard microscope (such as a standard inverted microscope) due to the short distance between the tissue membrane and the well surface. A stereomicroscope

can also be used to image the tissue from above, due to the large grid design of the membrane holder that does not cover much of the tissue membrane.

To monitor the growth of the tissue (such as cartilage tissue), one can perform any standard *in vitro* assay, such as the metabolic activity (MTT/Presto Blue) or live/dead assay, directly on the tissue membrane without removing it from the membrane holder. For assays that require extraction of the tissue (such as cartilage tissue) (e.g., gene expression assay), the membrane holder can be disassembled, and a small sample can be taken for analysis. However, membrane holding device should not be disassembled during the first week of culture, during which the cell spheroids have not fused together and may come off the mesh during the disassembling. After one week, the cell spheroids should have fused into a large tissue membrane, and it can be safely removed from the membrane holder and may continue to be cultured without the membrane holder.

Once the tissue membrane (such as cartilage tissue membrane) is matured, it can be used immediately for applications that require live cells. Alternatively, the tissue membrane can be processed further to modify the characteristic of the tissue (such as cartilage tissue). For example, the tissue membrane (such as cartilage tissue membrane) can be devitalized by boiling, freeze-thawing, or freeze-drying to eradicate living cells. The tissue membrane (such as cartilage tissue membrane) can also be decellurized using chemicals such as sodium dodecyl sulfate (SDS) to completely remove cellular substances to reduce the immunogenicity of the tissue membrane. The choice of processing depends on the final application for the tissue membrane (such as cartilage tissue membrane) and should be optimized by the users. For example, the inventors of the present disclosure developed the tissue membrane (such as cartilage tissue membrane) into a hypertrophic cartilage tissue for application in bone fracture healing (see Example 1 below).

Example 1: Making a hypertrophic cartilage membrane for bone fracture healing

Bone fracture healing occurs through the mechanism of endochondral ossification, in which a hypertrophic cartilage tissue (called the callus) is formed first and progressively calcified into bone tissue. Thus, it is hypothesized that by supplementing a hypertrophic cartilage membrane during bone fracture healing, the healing process could be enhanced.

To produce the hypertrophic cartilage membrane, bone marrow-derived human mesenchymal stem cells (hMSCs) was used (purchased commercially from Lonza, USA). Cells were expanded in proliferation medium consisting of DMEM low glucose, FBS 10%, pen-strep 1%, heparin 5 µg/mL and fibroblast growth factor 10 ng/mL. The cells were harvested at P6 and was made into cell spheroids (about 5,000 cells/spheroid) using the microwell method. A microwell plate can produce 1,000 spheroids per well on a standard 6-well plate. To make a 0.5×1 cm cartilage membrane, about 2,000 cell spheroids (10 million cells) are needed, which can be made from 2 wells of the 6-well plate.

After two days on the microwell plate, the cell spheroids formed. The cell spheroids were harvested and assembled between two Nylon meshes supported by a 6-well-size membrane holder (see FIG. 4A). The Nylon mesh (pore size: 250×300 µm, Simport, USA) was cut out from a large sheet to fit the membrane holder's size and was sterilized by autoclaving. The membrane holder) was 3D-printed (PLA material) and was soaked in 70% ethanol for sterilization. After being assembled, the membrane holder with its tissue membrane inside was cultured in hMSC chondrogenic medium consisting of DMEM high glucose, penstrep 1%, insulin-transferrin-selenium 1%, ascorbic acid 200 µM, proline 40 µg/mL and transforming growth factor beta 10 ng/mL. Medium was changed every 2-3 days, and the tissue membrane was cultured for 14-21 days for cartilage development.

To monitor the culture process, regular imaging was performed using a standard inverted microscope to check the status of the cell spheroids and live/dead staining was performed to evaluate the viability of the cells (see FIG. 5A to FIG. 5D). During the first few days of culture, the spheroids were still

separated and visible as individual spheroids (see FIG. 6A). After one week, most of the spheroids had fused into one tissue layer (see FIG. 6B). Depending on the initial seeding density, the tissue layer can be partially or fully covered by spheroids. The final cartilage tissue membrane consisted of a cartilage tissue
5 sandwiched between two layers of Nylon mesh (see FIG. 6C).

To characterize for cartilage development, the cartilage tissue was sampled after 14 days in culture to analyze for gene expression by quantitative polymerase chain reaction (qPCR). One whole tissue membrane was collected
10 for this analysis. Alternatively, one portion of the tissue membrane can be collected (e.g., by cutting using a sterile pair of scissors) for analysis. Standard qPCR analysis using commercial human gene expression assays (TaqMan, ThermoFisher) on a real-time PCR system (Biorad CFX96) was performed. Overall, the tissue membranes showed the typical gene expression profile of a
15 successfully developed cartilage tissue (see FIG. 7). Chondrogenic transcription factor SOX9 was upregulated 4-8 times versus Day 0 (before starting chondrogenic medium). Cartilage matrix protein collagen type 2 (COL2A) was upregulated 150,000 – 250,000 times and aggrecan (ACAN) 60-100 times. Hypertrophic cartilage transcription factors (RUNX2 and SP7) both increased 8
20 to 30 times. Hypertrophic cartilage matrix protein collagen type 1 (COL1A) and collagen type 10 (COL10A) increased 16 times and 4,000 times, respectively.

Further optimizations were performed to enhance the chondrogenic differentiation of hMSCs on the membrane holder. First, different cell numbers
25 per cell spheroid were tested to generate the optimal building blocks for the cartilage tissue membrane (see FIG. 8). It was found that 5,000 cells per cell spheroid was optimum for hMSCs differentiation into a cartilage tissue membrane on the membrane holder.

30 Next, the time when the spheroids should be transferred from the microwell plates onto the membrane holders was optimized (see FIG. 9). Although cell spheroids can form as soon as four hours after seeding, it was

observed that keeping the cell spheroids on microwell plates for a minimum of two days significantly enhances cartilage development.

Finally, to confirm the gene expression data, a histological analysis of the cartilage tissue membrane was performed. After 21 days in culture, the tissue membrane (10×5×0.5 mm) was harvested and processed for histological analysis. The tissue membrane was longitudinally cut to show all the layers and stained with Alcian Blue which is specific for proteoglycans and hyaluronic acid in cartilage tissue. Overall, the tissue membrane was homogeneously stained with Alcian Blue over its entire 10-mm length (see FIG. 10). Under high magnification, abundant extracellular matrix (ECM) and large round cell lacunae which is the typical morphology of cartilage tissue were observed. Some areas of terminally differentiated hypertrophic chondrocytes were evidenced by the fading blue stain. Terminal hypertrophic chondrocytes undergo apoptosis and release a plethora of growth factors, which is the normal process for endochondral ossification. The hypertrophic cartilage at this stage is the ideal template for bone regeneration.

The cartilage tissue membrane can be used directly (with live cells) as an allogeneic cell therapy treatment. It can also be processed by various means such as devitalizing (e.g., boiling, freeze-thawing, freeze-drying) to eradicate the cells or decellularizing (e.g., SDS) to remove cellular components and reduce the immunogenicity of the tissue before implantation. Furthermore, the hypertrophic cartilage material, which is rich in collagen type 1, 2 and 10, can be extracted using acid or enzymatic extraction methods. This hypertrophic cartilage cell-derived matrix (CDM) material can be used to formulate sponge/hydrogel/aerogel scaffolds for research/clinical applications.

While the above example describes the production of a cartilage tissue membrane, it will be appreciated that the technology of the present disclosure is also applicable to produce other tissue types. In general, any tissue that can be tissue-engineered using cell spheroids is applicable to this technology. This

include examples such as but are not limited to skin, muscle, vascular, pancreas, heart, neural tissue, and the like. The tissue membranes produced by the technology of the present disclosure can be widely utilized for research (e.g., tissue models for drug testing, biomaterials) or therapeutics (e.g., implantable tissue/scaffolds).

Example 2: Producing a cartilage membrane for cartilage repair

Spheroids consisting of about 5,000 cells (hMSCs, Lonza) were assembled between two Nylon meshes on a membrane holding device (MHD) with a 6-mm membrane diameter (see FIG. 11A). The membrane holding device and tissue membrane construct were cultured in chondrogenic medium *in vitro* for 14 days and thereafter harvested for testing (see FIG. 11B).

Histological images of the cartilage membrane showed a smooth and even surface, with abundant extracellular matrix and collagen type 2, resembling hyaline cartilage tissue (see FIG. 11C to FIG. 11E). SEM images revealed a cartilage-like matrix with abundant extracellular matrix and large round cell lacunae (see FIG. 11F and FIG. 11G).

Mechanical testing of the cartilage membrane was performed. A 5-mm diameter sample of the cartilage membrane was prepared by punching before the mechanical test (see FIG. 12A). A mechanical test setup was used to perform the mechanical testing (see FIG. 12B). The sample of cartilage membrane was compressed while being soaked in buffer (such as PBS) on a dish (such as glass petri dish). Equilibrium compression of the cartilage membranes was measured during an unconfined compression test. The equilibrium modulus as shown on the right-hand-side of FIG. 12D was determined from the linear region of the compression (10-20% strain). The mean modulus of the two samples is 21.5 kPa, compared to about 0.5-2.5 MPa of native human cartilage tissue, suggesting that chondrogenic differentiation of hMSCs with growth factor alone is not sufficient for obtaining optimal mechanical property.

Biocompatibility of the cartilage tissue membrane *in vivo* was also tested. Cartilage membranes were constructed from hMSCs (Lonza) and cultured for 2 weeks in chondrogenic medium before being subcutaneously implanted in rats (IACUC-231756, BRC-A*STAR). After 2 weeks of culture *in vivo*, the tissue membrane integrated into the subcutaneous layer of the rat skin. Histological slides, stained with H&E (FIG. 13A) and Alcian blue (FIG. 13B) revealed distinct regions of both rat and human tissue, with the human cells remaining viable. This experiment demonstrates the viability of the cartilage tissue membrane for two weeks in immunocompetent rats.

Comparative Example 1: Producing a tissue membrane from single cell suspension

Single cell suspensions of different densities (30,000 cells/cm², 300,000 cells/cm², 600,000 cells/cm², 900,000 cells/cm²) were seeded on Nylon meshes to investigate tissue membrane formation from single cell suspension in the same chondrogenic medium. Microscopy images of the different groups in this comparative example showed that the cells seeded from single cell suspensions did not successfully form cartilage tissue membranes (see FIG. 14). Although some preliminary tissue was formed, the tissue did not differentiate successfully into cartilage tissue, as shown by the reduction in cartilage gene markers (see qPCR data in FIG. 15). In particular, the expression of collagen type 2 (which is crucial for cartilage matrix) was not detectable at all.

APPLICATIONS

Embodiments of the present disclosure provide a membrane holding device for producing a tissue membrane, a method of producing a tissue membrane, and a tissue membrane construct for producing the tissue membrane, e.g., a biomimetic cartilage tissue membrane *in vitro*. Embodiments

of the disclosed device and methods also seek to overcome the problems of spheroids clumping into a thick structure, non-uniform tissue membrane development, medium perfusion and diffusion issues, poor mechanical strength, and relatively high cost versus low throughput of 3D printing equipment for assembling spheroids.

Embodiments of the disclosed method may advantageously assemble spheroids in a relatively large and thin membrane, supported by two meshes for maximum medium diffusion through the thin surface of the tissue membrane.

Embodiments of the disclosed method advantageously provide a relatively fast, simple assembling method and does not require expensive equipment. Even more advantageously, the tissue membrane size, shape and thickness may be easily tunable. Embodiments of the disclosed method advantageously use meshes that provide excellent mechanical strength, thus making the tissue membrane suitable for orthopaedic applications.

Embodiments of the disclosed membrane holding device advantageously work well with the disclosed method. Embodiments of the disclosed device are designed to press down firmly and evenly on the meshes, exerting sufficient compression to straighten the meshes, thus constraining the spheroids into a thin tissue membrane. Embodiments of the disclosed device are designed for easy assembling of the spheroids by providing a mechanism for seeding the spheroids evenly on the mesh. Embodiments of the disclosed device are designed for easy tuning of the size, shape and thickness for the tissue membrane.

It will be appreciated by a person skilled in the art that other variations and/or modifications may be made to the embodiments disclosed herein without departing from the spirit or scope of the disclosure as broadly described. For example, in the description herein, features of different exemplary embodiments may be mixed, combined, interchanged, incorporated, adopted, modified, included etc. or the like across different exemplary embodiments. The present

embodiments are, therefore, to be considered in all respects to be illustrative and not restrictive.

CLAIMS

1. A membrane holding device for producing a tissue membrane, the device comprising,
- 5 a first plate comprising a plurality of holes; and
a second plate comprising a plurality of holes, said second plate configured to be detachably coupled to the first plate;
wherein the first and second plates are configured to exert a substantially even compression on a tissue membrane construct held therebetween, when
10 coupled together.
2. The membrane holding device of claim 1,
wherein the first plate comprises a first grid with a network of grid bars defining the plurality of holes;
- 15 wherein the second plate comprises a second grid with a network of grid bars defining the plurality of holes; and
wherein the first and second grids are configured to be aligned when the first and second plates are coupled together.
- 20 3. The membrane holding device of claim 2, wherein the grid bars have an average thickness falling in the range of from 0.1 mm to 10 mm.
4. The membrane holding device of claim 2 or 3, wherein for each of the first and second plates, the ratio of area occupied by the network of grid bars
25 to the total area of the grid falls in the range of from 1% to 60%.
5. The membrane holding device of any one of claims 1 to 4, wherein each hole in the plurality of holes has dimensions of 5 mm by 5 mm to 50 mm by 50 mm.
- 30

6. The membrane holding device of any one of claims 1 to 5, wherein the plurality of holes have substantially the same dimensions and are spaced apart in a regular manner.

5 7. The membrane holding device of any one of claims 1 to 6, further comprising,

a first membrane with one side configured to contact the first plate and an opposite side configured to interface with a layer of cell spheroids; and

10 a second membrane with one side configured to contact the second plate and an opposite side configured to interface with the layer of cell spheroids.

8. The membrane holding device of claim 7, wherein the first and second membranes are in the form of meshes formed using threads having an average diameter of less than 200 μm .

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9. The membrane holding device of any one of claims 1 to 8, further comprising one or more coupling members configured for facilitating coupling of the first plate to the second plate,

20 wherein the one or more coupling members are further configured to allow adjustable control of the compression applied to the tissue membrane construct.

10. The membrane holding device of any one of claims 1 to 9, wherein the membrane holding device is stackable with a plurality of membrane holding devices to facilitate production of a plurality of tissue membranes.

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11. The membrane holding device of any one of claims 1 to 10, wherein the membrane holding device is configured to be integrated into a bioreactor system for facilitating transport of substances into and out of the tissue membrane construct.

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12. A method of producing a tissue membrane, the method comprising,

providing a membrane holding device comprising a first plate and a second plate configured to be detachably coupled to the first plate;

assembling a tissue membrane construct comprising a layer of cell spheroids disposed between a first membrane and a second membrane, such
5 that the tissue membrane construct is held in position between the first and second plates of the membrane holding device; and

maintaining the membrane holding device with the tissue membrane construct under culture conditions to produce the tissue membrane.

10 13. The method of claim 12, further comprising a step of seeding cell spheroids to form the layer of cell spheroids.

14. The method of claim 13, wherein the seeding step comprises,
placing the first membrane on the first plate;
15 placing the second plate relative to the first plate such that the first membrane is disposed between the first and second plates;
depositing cell spheroids onto the first membrane via a plurality of holes provided in the second plate to form the layer of cell spheroids;
removing the second plate;
20 positioning the second membrane over the layer of cell spheroids such that the layer of cell spheroids is disposed between the first and second membranes; and
coupling the first and second plates to each other.

25 15. The method of any one of claims 12 to 14,
wherein the first and second membranes comprise a plurality of pores, and
wherein the plurality of pores have an average pore size which is smaller than an average diameter of the cell spheroids.

30 16. The method of claim 15, wherein the plurality of pores of the first and second membranes have an average diameter falling in the range of from 0.1 μm to 500 μm .

17. The method of any one of claims 12 to 16, wherein the cell spheroids have an average diameter falling in the range of from 50 μm to 2000 μm .

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18. The method of any one of claims 12 to 17, wherein the cell spheroids comprise from 100 cells to 500,000 cells per spheroid.

19. The method of any one of claims 12 to 18, wherein the cell spheroids are seeded at a density falling in the range of from 500 to 10,000 cell spheroids per cm^2 .

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20. The method of any one of claims 12 to 19, wherein the tissue membrane has an average thickness falling in the range of from 50 μm to 2000 μm .

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21. A tissue membrane construct comprising,
a first membrane comprising a plurality of pores;
a second membrane comprising a plurality of pores; and
a layer of cell spheroids disposed between the first and second membranes.

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22. The tissue membrane construct of claim 21, wherein the tissue membrane construct is stackable with one or more additional tissue membrane constructs to form a thicker tissue construct.

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