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Description

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/511,948, filed July 26, 2011.

BACKGROUND OF THE INVENTION

[0002] Protein is a nutrient needed by the human body for growth and maintenance. Aside from water, protein is the most abundant molecule in the body. According to U.S. and Canadian Dietary Reference Intake guidelines, women aged 19-70 need to consume 46 grams of protein per day, while men aged 19-70 need to consume 56 grams of protein per day to avoid deficiency. This recommendation, however, is for a sedentary person free of disease. Protein deficiency can lead to reduced intelligence or mental retardation as well as contribute to the prevalence of diseases such as kwashiorkor. Protein deficiency is a serious problem in developing countries, particularly, in countries affected by war, famine, and overpopulation. Animal sources of protein, such as meat, are often a source of the complete complement of all the essential amino acids in adequate proportions.

[0003] The nutritional benefits of meat are tempered by potential associated environmental degradation. According to a 2006 report by the Livestock, Environment And Development Initiative, entitled Livestock's Long Shadow-Environmental Issues and Options, the livestock industry is one of the largest contributors to environmental degradation worldwide, and modern practices of raising animals for food contributes widely to air and water pollution, land degradation, climate change, and loss of biodiversity. The production and consumption of meat and other animal sources of protein is also associated with the clearing of rainforests and species extinction. Accordingly, there is a need for a solution to demands for alternative to meat produced from live animals.

[0004] Further, Hopkins and Dacey, "Vegetarian Meat: Could Technology Save Animals and Satisfy Meat Eaters?", J Agric Environ Ethics (2008) 21: 579-596, proposed that the nascent biotechnology of tissue culture, originally researched for medical applications, holds promise for those wishing to eat meat but not harm animals because meat could be grown in vitro without killing animals.

[0005] Both Yang et al., "Cell sheet engineering: Recreating tissues without biodegradable scaffolds", Biomaterials (2005) 26: 6415-6422, and Matsuda et al., "Tissue Engineering Based on Cell Sheet Technology", Adv. Mater. (2007) 19: 3089-3099, disclose using temperature-responsive culture dishes to achieve reversible cell adhesion to and detachment from dish surfaces, which allows for the non-invasive harvest of cultured cells as a monolayer cell sheet without the need for biodegradable scaffolds or the use of proteolytic enzymes. By avoiding the use of any additional materials such as carrier substrates or scaffolds, the complications associated with traditional tissue engineering approaches such as host inflammatory responses to implanted polymer materials, can be avoided. Thus, cell sheet engineering allows for tissue regeneration by either direct transplantation of cell sheets to host tissues or the creation of three-dimensional structures via the layering of individual cell sheets.

[0006] Sekine et al., "Myocardial tissue reconstruction: The cell sheet engineering approach", Inflammation and Regeneration (2007) 27(3): 171-176, also proposed cell sheet-based tissue engineering, which involves stacking confluently cultured cell sheets to construct 3-D cell-dense tissues. Upon layering, individual cardiomyocyte sheets integrate to form a single, continuous, cell-dense tissue that resembles native cardiac muscle. When transplanted directly to host hearts, these engineered myocardial tissues are able to form morphological connections to the host with the presence of functional gap junctions. Sekine et al. attempted to promote neovascularization within bioengineered myocardial tissues to overcome the longstanding limitations on engineered tissue thickness. As a possible advanced therapy, Sekine et al. attempted to fabricate functional myocardial tubes which may have the potential for circulatory support.

[0007] Aldhous, "Print me a heart and a set of arteries", New Scientist (15 April 2006) p.19 reported on bioprinting by using droplets of clumps of chicken heart cells that were a few hundred micrometers in diameter and allowed to flow together and fuse with alternate layers of supporting gel to form layers, rings or other shapes of cells that appear to function normally.

[0008] In U.S. Pat. App. 20050084958, Vein et al, disclosed a non-human tissue engineered meat product and a method for producing such meat product. The meat product comprised muscle cells that were grown ex vivo and used for food consumption. The muscle cells could be grown and attached to a support structure and could be derived from any non-human cells. The meat product could also comprise other cells such as fat cells or cartilage cells, or both, that were grown ex vivo together with the muscle cells.

[0009] In U.S. Pat. App. 20100041134, Forgacs et al., disclose structures and methods for tissue engineering that include a multicellular body including a plurality of living cells. Forgacs et al. disclose that a plurality of multicellular bodies can be arranged in a pattern and allowed to fuse to form an engineered tissue. The arrangement can include filler bodies including a biocompatible material that resists migration and ingrowth of cells from the multicellular bodies and that is resistant to adherence of cells to it. Forgacs et al. disclose that three-dimensional constructs can be assembled by printing or otherwise stacking the multicellular bodies and filler bodies such that there is direct contact between adjoining multicellular bodies, suitably along a contact area that has a substantial length. The direct contact between the multicellular bodies promotes efficient and reliable fusion. The increased contact area between adjoining multicellular

bodies also promotes efficient and reliable fusion. Forgacs et al. also disclose methods of producing multicellular bodies having characteristics that facilitate assembly of the three-dimensional constructs.

SUMMARY OF THE INVENTION

[0010] Tissue engineering technology offers new opportunities to produce edible sources of animal protein that are not associated with the environmental degradation of raising livestock. Tissue engineering has been defined as an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ. Langer R, Vacanti JP, Tissue Engineering, Science 260(5110):920-926 (May 1993). Despite the potential to apply tissue engineering technology to meet the nutritional needs of living beings, scientifically sound and industrially feasible processes have not been developed to produce comestible meat and engineered comestible meat products are not available.

[0011] The invention provides a method of forming engineered meat, as defined by the claims appended to this description.

[0012] A method of forming engineered meat, the method comprising:

preparing a plurality of multicellular bodies comprising a plurality of non-human myocytes cohered to one another;
laying more than one multicellular body adjacently onto a planar support substrate;
fusing said multicellular bodies at least partially together to form a first layer;
stacking more than 50 additional layers onto the first layer;
fusing the stacked layers to form a volume of engineered meat; and
culturing the stacked layers to fuse the layers while the layers in an inner region of the volume die such that the majority of cells in the volume have died after fusion between the layers is at least partially complete, and
wherein the engineered meat is comestible.

[0013] As stated above, the method of the invention optionally includes a step of preparing the plurality of multicellular bodies by culturing a plurality of non-human myocyte cells and non-human endothelial cells at least until the cells are cohered to one another. As mentioned above, any other appropriate non-human cell type may be included as part of some or all of the multicellular bodies forming the layers, including endothelial cells and/or adipose cells, and/or fibroblast cells.

[0014] During the formation of the engineered meat product, the layers maybe individually or collectively stacked atop other layer to create the volume of engineered meat. In some variations each successive layer is differently oriented with respect to the adjacent layer(s). For example, as they are stacked, the new layers may be rotated relative to the other layers in the volume. In some variations, each layer is rotated approximately 90° relative to the other layers as it is stacked.

[0015] In any of the engineered meat described herein the layers may be exercised as they are formed. As described in greater detail below, exercising the layers may enhance the formation of extracellular matrix (ECM). This may also orient the cells (e.g., myocytes) within a layer as it is formed. Thus, in some variations of the method of forming the engineered meat may include a step of applying mechanical, electrical or electromechanical force to exercise the myocytes in each layer.

[0016] As mentioned, the step of stacking the layers includes stacking more than 50 layers, more than about 100 layers, or the like.

[0017] In some embodiments, the methods provided herein further comprise freezing said meat.

[0018] In a another aspect, disclosed herein are methods of forming engineered meat, comprising: preparing a plurality of elongate multicellular bodies comprising a plurality of living non-human myocytes wherein the cells are adhered and/or cohered to one another; preparing a plurality of substantially spherical multicellular bodies comprising a plurality of living non-human myocytes wherein the cells are adhered and/or cohered to one another; laying more than one elongate multicellular body and more than one substantially spherical multicellular body adjacently onto a support substrate; allowing said multicellular bodies to fuse to form a layer; laying (e.g., stacking) more than 50 layers onto the first layer; allowing said layers to fuse to form engineered meat; and optionally, freezing said meat; provided that the engineered meat is comestible and for ingestion. In some embodiments, disclosed herein are methods of forming engineered meat, comprising: preparing a plurality of elongate multicellular bodies comprising a plurality of non-human myocytes wherein the cells are adhered and/or cohered to one another; preparing a plurality of substantially spherical multicellular bodies comprising a plurality of non-human myocytes wherein the cells are adhered and/or cohered to one another; laying more than one elongate multicellular body and more than one substantially spherical multicellular body adjacently onto a support substrate; fusing said multicellular bodies to form a layer; laying more than 50 layers onto the first layer; and fusing said layers to form a volume of engineered meat; provided that the engineered meat is comestible and for ingestion. In some embodiments, the methods provided herein further comprise freezing said meat.

[0019] The ratio of the elongate multicellular bodies and the substantially spherical multicellular bodies can be about 0:100, 1:100, 2:100, 3:100, 4:100, 5:100, 6:100, 7:100, 8:100, 9:100, 1:10, 11:100, 12:100, 13:100, 14:100, 15:100, 16:100, 17:100, 18:100, 19:100, 1:5, 21:100, 22:100, 23:100, 24:100, 25:100, 26:100, 27:100,

28:100, 29:100, 3:10, 31:100, 32:100, 33:100, 34:100, 35:100, 36:100, 37:100, 38:100, 39:100, 2:5, 41:100, 42:100, 43:100, 44:100, 45:100, 46:100, 47:100, 48:100, 49:100, 1:2, 51:100, 52:100, 53:100, 54:100, 55:100, 56:100, 57:100, 58:100, 59:100, 3:5, 61:100, 62:100, 63:100, 64:100, 65:100, 66:100, 67:100, 68:100, 69:100, 7:10, 71:100, 72:100, 73:100, 74:100, 75:100, 76:100, 77:100, 78:100, 79:100, 4:5, 81:100, 82:100, 83:100, 84:100, 85:100, 86:100, 87:100, 88:100, 89:100, 9:10, 91:100, 92:100, 93:100, 94:100, 95:100, 96:100, 97:100, 98:100, 99:100, or 1:1. The ratio of the substantially spherical multicellular bodies and the elongate multicellular bodies can be about 0:100, 1:100, 2:100, 3:100, 4:100, 5:100, 6:100, 7:100, 8:100, 9:100, 1:10, 11:100, 12:100, 13:100, 14:100, 15:100, 16:100, 17:100, 18:100, 19:100, 1:5, 21:100, 22:100, 23:100, 24:100, 25:100, 26:100, 27:100, 28:100, 29:100, 3:10, 31:100, 32:100, 33:100, 34:100, 35:100, 36:100, 37:100, 38:100, 39:100, 2:5, 41:100, 42:100, 43:100, 44:100, 45:100, 46:100, 47:100, 48:100, 49:100, 1:2, 51:100, 52:100, 53:100, 54:100, 55:100, 56:100, 57:100, 58:100, 59:100, 3:5, 61:100, 62:100, 63:100, 64:100, 65:100, 66:100, 67:100, 68:100, 69:100, 7:10, 71:100, 72:100, 73:100, 74:100, 75:100, 76:100, 77:100, 78:100, 79:100, 4:5, 81:100, 82:100, 83:100, 84:100, 85:100, 86:100, 87:100, 88:100, 89:100, 9:10, 91:100, 92:100, 93:100, 94:100, 95:100, 96:100, 97:100, 98:100, 99:100, or 1:1.

[0020] In a another aspect, disclosed herein are methods of forming engineered meat, comprising: preparing a plurality of substantially spherical multicellular bodies comprising a plurality of living non-human myocytes wherein the cells are adhered and/or cohered to one another; laying more than one substantially spherical multicellular body adjacently onto a support substrate; allowing said substantially spherical multicellular bodies to fuse to form a layer; laying more than 50 layers onto the first layer; allowing the layers to fuse to form a volume of engineered meat; and optionally, freezing said meat; provided that the engineered meat is comestible and for ingestion. In some embodiments, disclosed herein are methods of forming engineered meat, comprising: preparing a plurality of substantially spherical multicellular bodies comprising a plurality of non-human myocytes wherein the cells are adhered and/or cohered to one another; laying more than one substantially spherical multicellular body adjacently onto a support substrate; fusing said substantially spherical multicellular bodies to form a layer; laying more than 50 layers onto the first layer; and fusing said layers to form a volume of engineered meat; provided that the engineered meat is comestible. In some embodiments, the methods provided herein further comprise freezing said meat.

[0021] In some embodiments, the methods of forming engineered meat disclosed herein comprise preparing a plurality of multicellular bodies comprising a plurality of living non-human myocytes wherein the cells are adhered and/or cohered to one another, wherein the multicellular bodies further comprise living, non-human adipose cells, and/or endothelial cells. In some embodiments, the multicellular bodies further comprise living, non-human fibroblast cells. In some embodiments, the methods of forming engineered meat disclosed herein comprise laying more than one multicellular body adjacently onto a support substrate, wherein the multicellular bodies are laid horizontally adjacent and/or vertically adjacent. In some embodiments, the methods of forming engineered meat disclosed herein comprise laying more than one layer adjacently onto a support substrate, wherein the layers are laid horizontally adjacent and/or vertically adjacent. In some embodiments, the support substrate is permeable to fluids and nutrients and allows cell culture media to contact all surfaces of said multicellular bodies and/or layers. In some embodiments, the methods of forming engineered meat disclosed herein comprise allowing multicellular bodies to fuse to form a layer, wherein the multicellular bodies fuse to form a layer in a cell culture environment. In some embodiments, fusing of multicellular bodies takes place over about 2 hours to about 36 hours. In some embodiments, the methods comprise allowing layers to fuse to form engineered meat, wherein the layers fuse to form engineered meat in a cell culture environment. In some embodiments, fusing of layers takes place over about 2 hours to about 36 hours. In some embodiments, the elongate multicellular bodies of non-human myocytes and non-human endothelial cells are of differing lengths. In various embodiments, the elongate multicellular bodies have a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mm. In various embodiments, the elongate multicellular bodies have a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 cm. In some embodiments, the elongate multicellular bodies have a length ranging from 1 mm to 10 cm. In further embodiments, the elongate multicellular bodies have a length ranging from about 1 cm to about 8 cm. In still further embodiments, the elongate multicellular bodies have a length ranging from about 2 cm to about 6 cm. In some embodiments, the methods of forming engineered meat disclosed herein comprise laying more than one layer adjacently onto a support substrate and allowing the layers to fuse to form engineered meat. In various embodiments, the meat comprises 50, 60, 70, 80, 90, or 100 layers. In some embodiments, the methods of forming engineered meat disclosed herein comprise preparing a plurality of multicellular bodies comprising a plurality of living non-human myocytes wherein the cells are adhered and/or cohered to one another, wherein the multicellular bodies have a diameter adapted to allow diffusion to sufficiently support the maintenance and growth of the non-human myocytes and non-human endothelial cells in culture. In various embodiments, the multicellular bodies have a diameter of 100, 200, 300, 400, or 500 μm. In some embodiments, the multicellular bodies have a diameter of 100 μm to 500 μm. In further embodiments, the multicellular bodies have a diameter of about 200 μm to about 400 μm. In some embodiments, the diameter applies to multicellular bodies with substantially rod or sphere shape. In some

embodiments, the methods of forming engineered meat disclosed herein comprise preparing a plurality of multicellular bodies comprising a plurality of living non-human myocytes wherein the cells are adhered and/or cohered to one another, wherein the multicellular bodies are bioprinted.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022]

Fig. 1 depicts a non-limiting example of a multicellular body; in this case, a multicellular body **1** with width W**1** that is approximately equal to height H**1** and length L**1** that is substantially greater than width W**1** or height H**1**.

Fig. 2 depicts a non-limiting example of a substantially spherical multicellular body; in this case, a substantially spherical multicellular body **2** with width W**1** that is approximately equal to height H**1**.

Fig. 3 depicts a non-limiting example of a multicellular body; in this case, a multicellular body **1** on a support substrate **3.**

Fig. 4 depicts a non-limiting example of a substantially spherical multicellular body; in this case, a substantially spherical multicellular body **2** on a support substrate **3.**

Fig. 5 depicts a non-limiting example of one method of making the multicellular bodies illustrated in Figs. **1-4;** in this case, a method involving transferring a mixed cell pellet **4** into a capillary tube **5.**

Fig. 6 depicts a non-limiting example of a plurality of multicellular bodies; in this case, a plurality of multicellular bodies **1** is laid adjacently onto a support substrate **3** such that they are allowed to fuse.

Fig. 7 depicts a non-limiting example of a plurality of substantially spherical multicellular bodies; in this case, a plurality of substantially spherical multicellular bodies **2** is laid adjacently onto a support substrate **3** such that they are allowed to fuse.

Fig. 8 depicts a non-limiting example of one method of making a layer comprising a plurality of multicellular bodies; in this case, a method involving extruding multicellular bodies **6** from a pressure-operated mechanical extruder comprising a capillary tube **5** onto a support substrate **3.**

Fig. 9 depicts a non-limiting example of one method of making engineered meat; in this case, a method involving laying more than one layer, comprising a plurality of multicellular bodies **7, 8,** adjacently onto a support substrate **3.**

Fig. 10 depicts a non-limiting example of one method of making engineered meat; in this case, a method involving laying more than one layer, comprising a plurality of multicellular bodies **9** and a plurality of substantially spherical multicellular bodies **10,** adjacently onto a support substrate **3.**

Fig. 11 depicts a non-limiting example of one method of making engineered meat; in this case, a method involving stacking more than one layer, wherein layers subsequent to the first are rotated 90 degrees with respect to the layer below.

DETAILED DESCRIPTION OF THE INVENTION

[0023] Tissue engineered products made using traditional materials and methods are limited in size due to the short distances gases and nutrients can diffuse to nourish interior cells. Also, existing techniques fail to provide adequate speed and throughput for mass production of engineered products. As a result, existing tissue engineering methods, used to produce meat products, result in unappealing thin sheets and pastes on a commercially infeasible scale.

[0024] Thus, an objective of the methods of making a comestible meat product disclosed herein is to provide commercially viable and appealing meat products. Another objective is to provide high-throughput methods that reliably, accurately, and reproducibly scale up to commercial levels. Advantages of the methods of making the comestible meat products disclosed herein include, but are not limited to, production of customized tissues in a reproducible, high throughput and easily scalable fashion while keeping precise control of pattern formation, particularly in cases of multiple cell types, which may result in engineered meat products with appealing flavor, texture, thickness, and appearance.

[0025] Provided herein, in various embodiments, are methods of forming engineered meat, the method comprising: a) preparing a plurality of multicellular bodies comprising a plurality of non-human myocytes cohered to one another; b) laying more than one multicellular body adjacently onto a planar support substrate; c) fusing said multicellular bodies at least partially together to form a first layer; d) stacking more than 50 additional layers onto the first layer; e) fusing the stacked layers to form a volume of engineered meat; and f) culturing the stacked layers to fuse the layers while the layers in an inner region of the volume die such that the majority of cells in the volume have died after fusion between the layers is at least partially complete, and wherein the engineered meat is comestible; and f) optionally, freezing said meat; provided that the engineered meat is comestible and for ingestion.

[0026] Also provided herein, in various embodiments, are methods of forming engineered meat, comprising: a)

preparing a plurality of elongate multicellular bodies and/or a plurality of substantially spherical multicellular bodies comprising a plurality of living non-human myocytes wherein the cells are adhered and/or cohered to one another; b) laying more than one elongate multicellular body and more than one substantially spherical multicellular body adjacently onto a support substrate; c) allowing said multicellular bodies to fuse to form a layer; d) stacking more than 50 additional layers onto the first layer; e) fusing the stacked layers to form a volume of engineered meat; and f) culturing the stacked layers to fuse the layers while the layers in an inner region of the volume die such that the majority of cells in the volume have died after fusion between the layers is at least partially complete, and wherein the engineered meat is comestible; and g) optionally, freezing said meat; provided that the engineered meat is comestible and for ingestion. Also disclosed herein, in various embodiments, are methods of forming engineered meat, comprising: a) preparing a plurality of elongate multicellular bodies and/or a plurality of substantially spherical multicellular bodies comprising a plurality of non-human myocytes wherein the cells are adhered and/or cohered to one another; b) laying more than one elongate multicellular body and more than one substantially spherical multicellular body adjacently onto a support substrate; c) fusing said multicellular bodies to form a layer; d) stacking more than one layer adjacently onto each other on a support substrate; and e) fusing said layers to form a volume of engineered meat; provided that the engineered meat is comestible. In some embodiments, the methods comprise laying more than one elongate multicellular body and more than one substantially spherical multicellular body in different ratios adjacently onto a support substrate. In some embodiments, the methods further comprise freezing said meat.

[0027] Also provided herein, in various embodiments, are methods of forming engineered meat, comprising: a) preparing a plurality of substantially spherical multicellular bodies comprising a plurality of living non-human myocytes wherein the cells are adhered and/or cohered to one another; b) laying more than one substantially spherical multicellular body adjacently onto a support substrate; c) allowing said substantially spherical multicellular bodies to fuse to form a layer; d) stacking more than about 50 additional layers onto the first layer; e) fusing the stacked layers to form a volume of engineered meat; and f) culturing the stacked layers to fuse the layers while the layers in an inner region of the volume die such that the majority of cells in the volume have died after fusion between the layers is at least partially complete, and wherein the engineered meat is comestible; and g) optionally, freezing said meat; provided that the engineered meat is comestible and for ingestion. Also disclosed herein, in various embodiments, are methods of forming engineered meat, comprising: a) preparing a plurality of substantially spherical multicellular bodies comprising a plurality of non-human myocytes wherein the cells are adhered and/or cohered to one another; b) laying more than one substantially spherical multicellular body adjacently onto a support substrate; c) fusing said substantially spherical multicellular bodies to form a layer; d) stacking more than about 50 additional layers onto the first layer; e) fusing the stacked layers to form a volume of engineered meat; and f) culturing the stacked layers to fuse the layers while the layers in an inner region of the volume die such that the majority of cells in the volume have died after fusion between the layers is at least partially complete, and wherein the engineered meat is comestible. In some embodiments, the methods further comprise freezing said meat.

[0028] A basic idea underlying classical tissue engineering is to seed living cells into biocompatible and eventually biodegradable scaffold, and then culture the system in a bioreactor so that the initial cell population can expand into a tissue. Classical tissue engineering harbors several shortcomings, especially when applied to the production of meat products. First, the process of seeding cells generally involves contacting a solution of cells with a scaffold such that the cells are trapped within pores, fibers, or other microstructure of the scaffold. This process is substantially random with regard to the placement of cells within the scaffold and the placement of cells relative to each other. Therefore, seeded scaffolds are not immediately useful for production of three-dimensional constructs that exhibit planned or pre-determined placement or patterns of cells or cell aggregates. Second, selection of the ideal biomaterial scaffold for a given cell type is problematic and often accomplished by trial and error. Even if the right biomaterial is available, a scaffold can interfere with achieving high cell density. Moreover, scaffold-based tissue engineering does not easily or reliably scale up to industrial levels.

[0029] In some embodiments, the engineered meat products, layers, and multicellular bodies, are made with a method that utilizes a rapid prototyping technology based on three-dimensional, automated, computer-aided deposition of multicellular bodies (e.g., cylinders and spheroids) and a biocompatible support structure (e.g., composed of agarose) by a three-dimensional delivery device (e.g., a bioprinter). The term "engineered," typically means man-made or arranged when used to refer to the layers and the meat products described herein. One example of an engineered meat may include arranging or placing multicellular bodies and/or layers to form engineered meat products by a computer-aided device (e.g., a bioprinter) according to a computer script. In further embodiments, the computer script is, for example, one or more computer programs, computer applications, or computer modules. In still further embodiments, three-dimensional tissue structures form through the post-printing fusion of the multicellular bodies similar to self-assembly phenomena in early morphogenesis.

[0030] Unlike other engineered tissues, the engineered meat described herein is formed by stacking layers of two-dimensional planar sheets of at least partially

fused multicellular bodies. Thus, methods for forming even large volumes of engineered meat described herein may not require simultaneous three dimensional patterning, but may be performed by culturing (in parallel) multiple two-dimensional layers that may be later assembled into a three-dimensional assembly, or sub-assemblies that can then be stacked together. This advantageous method of forming the engineered meats described herein may permit the volume of engineered meat to be formed without requiring the need for scaffolding or three-dimensional support structures, such as filler bodies. Further, the two-dimensional layers may be formed in parallel at a relatively thin thickness that allows for diffusion of nutrients from a culture medium into the planar layer during culture (e.g., while fusing the component multicellular bodies into the layer). It is only after the component layers are stacked to form the volume that diffusion of nutrients may be limiting, resulting in cell death.

[0031] Thus, while a number of methods are available to arrange the multicellular bodies on a support substrate to produce a three-dimensional structure including manual placement, including positioning by an automated, computer-aided machine such as a bioprinter, such methods may be useful but are not required. Advantages of delivery of multicellular bodies with bioprinter technology include rapid, accurate, and reproducible placement of multicellular bodies to produce constructs exhibiting planned or pre-determined orientations or patterns of multicellular bodies and/or layers of various compositions. Advantages also include assured high cell density, while minimizing cell damage often associated with other solid freeform fabrication-based deposition methods focused on printing cells in combination with hydrogels.

[0032] Also disclosed herein are methods of manufacture or making of engineered meats, and business methods. In some embodiments, the speed and scalability of the techniques and methods disclosed herein are utilized to design, build, and operate industrial and/or commercial facilities for the production of comestible, engineered meat products. In further embodiments, the engineered meat products are produced, packaged, frozen, stored, distributed, marketed, advertised, and sold as, for example, food products for human beings, components or ingredients of food products for human beings, food products for non-human animals, or components or ingredients of food products for non-human animals.

Cells

[0033] Many self-adhering cell types may be used to form the multicellular bodies, layers, and engineered meat products described herein. In some embodiments, the engineered meat products are designed to resemble traditional meat products and the cell types are chosen to approximate those found in traditional meat products. In further embodiments, the engineered meat products, layers, and multicellular bodies include non-human myocytes. In still further embodiments, the engineered meat products, layers, and multicellular bodies include non-human myocytes, and/or endothelial cells, and/or adipose cells, and/or fibroblasts.

[0034] In general, the engineered meats described herein may differ from natural meats and other engineered meats by lacking blood vessels, and also lacking in nerve enervation. Even in variations in which endothelial cells are included as a component of one or more multicellular body, the engineered meat will not include blood vessels competent to transmit blood. Thus, even the large volumes of engineered meat formed by the methods described herein may not have any blood vessels. Further, the engineered meats described herein may lack any nerve components (e.g., axons, dendrites, nerve cell bodies), as they may be grown without such components.

[0035] Human beings traditionally eat several types of animal muscle tissue. Therefore, in some embodiments, the myocytes are skeletal myocytes. In some embodiments, the myocytes are cardiac myocytes. In some embodiments, the myocytes are smooth myocytes. In some embodiments, the endothelial cells are microvascular endothelial cells.

[0036] In other embodiments, the engineered meat products include neural cells, connective tissue (including bone, cartilage, cells differentiating into bone forming cells and chondrocytes, and lymph tissues), epithelial cells (including endothelial cells that form linings in cavities and vessels or channels, exocrine secretory epithelial cells, epithelial absorptive cells, keratinizing epithelial cells, and extracellular matrix secretion cells), and undifferentiated cells (such as embryonic cells, stem cells, and other precursor cells), among others.

[0037] In some embodiments, the cells used to form a multicellular body are obtained from a live animal and cultured as a primary cell line. For example, in further embodiments, the cells are obtained by biopsy and cultured *ex vivo.* In other embodiments, the cells are obtained from commercial sources.

[0038] The engineered meat products and the layers comprising a plurality of multicellular bodies for use in production of said meat disclosed herein are comestible and intended for consumption by human beings, non-human animals, or both. In some embodiments, the engineered meat products are human food products. In other embodiments, the engineered meat products are animal feed such as feed for livestock, feed for aquaculture, or feed for domestic pets. Therefore, in light of the disclosure provided herein, those of skill in the art will recognize that non-human cells from a plethora of sources are suitable for use in production of such products and with the methods disclosed herein. In various embodiments, the multicellular bodies, layers comprising multicellular bodies, and engineered meat products comprise non-human cells derived from, by way of non-limiting examples, mammals, birds, reptiles, fish, crustaceans, mollusks, cephalopods, insects, non-arthropod invertebrates, and combinations thereof.

[0039] In some embodiments, suitable cells are derived from mammals such as antelope, bear, beaver, bison, boar, camel, caribou, cattle, deer, elephant, elk, fox, giraffe, goat, hare, horse, ibex, kangaroo, lion, llama, moose, peccary, pig, rabbit, seal, sheep, squirrel, tiger, whale, yak, and zebra, or combinations thereof. In some embodiments, suitable cells are derived from birds such as chicken, duck, emu, goose, grouse, ostrich, pheasant, pigeon, quail, and turkey, or combinations thereof. In some embodiments, suitable cells are derived from reptiles such as turtle, snake, crocodile, and alligator, or combinations thereof. In some embodiments, suitable cells are derived from fish such as anchovy, bass, catfish, carp, cod, eel, flounder, fugu, grouper, haddock, halibut, herring, mackerel, mahi mahi, marlin, orange roughy, perch, pike, pollock, salmon, sardine, shark, snapper, sole, swordfish, tilapia, trout, tuna, and walleye, or combinations thereof. In some embodiments, suitable cells are derived from crustaceans such as crab, crayfish, lobster, prawn, and shrimp, or combinations thereof. In some embodiments, suitable cells are derived from mollusks such as abalone, clam, conch, mussel, oyster, scallop, and snail, or combinations thereof. In some embodiments, suitable cells are derived from cephalopods such as cuttlefish, octopus, and squid, or combinations thereof. In some embodiments, suitable cells are derived from insects such as ants, bees, beetles, butterflies, cockroaches, crickets, damselflies, dragonflies, earwigs, fleas, flies, grasshoppers, mantids, mayflies, moths, silverfish, termites, wasps, or combinations thereof. In some embodiments, suitable cells are derived from non-arthropod invertebrates (e.g., worms) such as flatworms, tapeworms, flukes, threadworms, roundworms, hookworms, segmented worms (e.g., earthworms, bristle worms, etc.), or combinations thereof.

Multicellular bodies

[0040] Disclosed herein are multicellular bodies including a plurality of living non-human cells wherein the cells are adhered and/or cohered to one another. Also disclosed herein are methods comprising: preparing a plurality of multicellular bodies comprising a plurality of living non-human myocytes wherein the cells are adhered and/or cohered to one another; laying more than one multicellular body adjacently onto a support substrate; and allowing the multicellular bodies to fuse to form a substantially planar layer for used in forming engineered meat. In some embodiments, a multicellular body comprises a plurality of cells adhered and/or cohered together in a desired three-dimensional shape with viscoelastic consistency and sufficient integrity for easy manipulation and handling during a bioengineering process, such as tissue engineering. In some embodiments, sufficient integrity means that the multicellular body, during the subsequent handling, is capable of retaining its physical shape, which is not rigid, but has a viscoelastic consistency, and maintaining the vitality of the cells.

[0041] In some embodiments, a multicellular body is homocellular. In other embodiments, a multicellular body is heterocellular. In homocellular multicellular bodies, the plurality of living cells includes a plurality of living cells of a single cell type. Substantially all of the living cells in a homocellular multicellular body are substantially cells of the single cell type. In contrast, a heterocellular multicellular body includes significant numbers of cells of more than one cell type. The living cells in a heterocellular body may remain unsorted or can "sort out" (e.g., self-assemble) during the fusion process to form a particular internal structure for the engineered tissue. The sorting of cells is consistent with the predictions of the Differential Adhesion Hypothesis (DAH). The DAH explains the liquid-like behavior of cell populations in terms of tissue surface and interfacial tensions generated by adhesive and cohesive interactions between the component cells. In general, cells can sort based on differences in the adhesive strengths of the cells. For example, cell types that sort to the interior of a heterocellular multicellular body generally have a stronger adhesion strength (and thus higher surface tension) than cells that sort to the outside of the multicellular body.

[0042] In some embodiments, the multicellular bodies disclosed herein also include one or more extracellular matrix (ECM) components or one or more derivatives of one or more ECM components in addition to the plurality of cells. For example, the multicellular bodies may contain various ECM proteins including, by way of non-limiting examples, gelatin, fibrinogen, fibrin, collagen, fibronectin, laminin, elastin, and proteoglycans. The ECM components or derivatives of ECM components can be added to a cell paste used to form a multicellular body. The ECM components or derivatives of ECM components added to a cell paste can be purified from an animal source, or produced by recombinant methods known in the art. Alternatively, the ECM components or derivatives of ECM components can be naturally secreted by the cells in the multicellular body.

[0043] In some embodiments, a multicellular body includes tissue culture medium. In further embodiments, the tissue culture medium can be any physiologically compatible medium and will typically be chosen according to the cell type(s) involved as is known in the art. In some cases, suitable tissue culture medium comprises, for example, basic nutrients such as sugars and amino acids, growth factors, antibiotics (to minimize contamination), etc.

[0044] The adhesion and/or cohesion of the cells in a multicellular body is suitably sufficiently strong to allow the multicellular body to retain a three-dimensional shape while supporting itself on a flat surface. For instance, in some cases, a multicellular body supporting itself on a flat substrate may exhibit some minor deformation (e.g., where the multicellular body contacts the surface), however, the multicellular body is sufficiently cohesive to retain a height that is at least one half its width, and in some cases, about equal to the width. In some embodiments,

two or more multicellular bodies placed in side-by-side adjoining relation to one another on a flat substrate form a void space under their sides and above the work surface. *See,* e.g., **Figs. 3** and **4.** In further embodiments, the cohesion of the cells in a multicellular body is sufficiently strong to allow the multicellular body to support the weight of at least one similarly sized and shaped multicellular body when the multicellular body is assembled in a construct in which the multicellular bodies are stacked on top of one another. *See,* e.g., **Figs. 9** and **10.** In still further embodiments, the adhesion and/or cohesion of the cells in a multicellular body is also suitably sufficiently strong to allow the multicellular body to be picked up by an implement (e.g., a capillary micropipette).

[0045] In light of the disclosure provided herein, those of skill in the art will recognize that multicellular bodies having different sizes and shapes are within the scope of the embodiments provided herein. In some embodiments, a multicellular body is substantially cylindrical and has a substantially circular cross section. For example, a multicellular body, in various embodiments, has an elongate shape (e.g., a cylindrical shape) with a square, rectangular, triangular, or other non-circular cross-sectional shape. Likewise, in various embodiments, a multicellular body has a generally spherical shape, a non-elongate cylindrical shape, or a cuboidal shape.

[0046] In various embodiments, the diameter of a multicellular body is about 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 μm, or quantifiable increments therein. In some embodiments, a multicellular body is configured to limit cell necrosis caused by inability of oxygen and/or nutrients to diffuse into central portions of the multicellular body. For example, a multicellular body is suitably configured such that none of the living cells therein is more than about 250 μm from an exterior surface of the multicellular body, and more suitably so none of the living cells therein is more than about 200 μm from an exterior of the multicellular body.

[0047] In some embodiments, the multicellular bodies have differing lengths. In other embodiments, multicellular bodies are of substantially similar lengths. In various embodiments, the length of a multicellular body is 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 mm, or quantifiable increments therein. In other various embodiments, the length of a multicellular body is a 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, or 10 cm, or quantifiable increments therein. In some embodiments, the length of multicellular bodies is chosen to result in a shape and/or size of engineered meat product that approximates that of a traditional meat product, for example, a strip of bacon, a hamburger patty, a fish fillet, a chicken breast, or a steak.

[0048] Referring to **Fig. 1**, in some embodiments, a multicellular body **1** is substantially cylindrical with a width **W1** roughly equal to a height **H1** and has a substantially circular cross section. In further embodiments, a multicellular body 1 is elongate with a length of **L1**. In still further embodiments, **W1** and **H1** are suitably about 300 to about 600 μm and **L1** is suitably about 2 cm to about 6 cm.

[0049] Referring to **Fig. 2,** in some embodiments, a multicellular body **2** is substantially spherical with a width **W1** roughly equal to a height **HI.** In further embodiments, **W1** and **H1** are suitably about 300 to about 600 μm.

Layers

[0050] The engineered meat obtained by the method according to the invention includes more than 50 layers on the first layer, each layer including a plurality of multicellular bodies comprising a plurality of living non-human cells wherein the cells are adhered and/or cohered to one another. Also disclosed herein are methods comprising the steps of laying multicellular bodies adjacently onto a support substrate and allowing the multicellular bodies to fuse to form a substantially planar layer for use in formation of engineered comestible meat products. In some embodiments, each layer is bioprinted, using techniques described herein.

[0051] In some embodiments, a layer includes homocellular multicellular bodies. In other embodiments, a layer includes heterocellular multicellular bodies. In yet other embodiments, a layer includes both homocellular and heterocellular multicellular bodies. In further embodiments, a layer includes non-human myocytes. In still further embodiments, a layer includes non-human myocytes, non-human endothelial cells, and adipose cells and/or fibroblast cells. In still further embodiments, a layer includes non-human myocytes, non-human endothelial cells, and other cell types disclosed herein.

[0052] In embodiments including both non-human myocytes and non-human endothelial cells, a layer may include non-human myocytes and non-human endothelial cells in a ratio of about 30:1, 29:1, 28:1, 27:1, 26:1, 25:1, 24:1, 23:1, 22:1, 21:1, 20:1, 19:1, 18:1, 17:1, 16:1, 15:1, 14:1, 13:1, 12:1, 11:1, 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, and 1:1, or increments therein. In some embodiments, a layer contains non-human myocytes and non-human endothelial cells in a ratio of about 19:1 to about 3:1. In various embodiments, a layer includes non-human endothelial cells that comprise about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, and 25%, or increments therein, of the total cell population. In some embodiments, a layer includes non-human endothelial cells that comprise about 5% to about 15% of the total cell population. In further embodiments, the presence of endothelial cells contributes to endothelialization, described further herein.

[0053] In various embodiments, the thickness of each layer is about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 2000, 3000, 4000, or 5000

μm, or quantifiable increments therein. In some embodiments, the thickness of each layer is chosen to allow diffusion to sufficiently support the maintenance and growth of substantially all the cells in the layer in culture.

[0054] In various embodiments, the plurality of layers includes about 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, or 500 layers, or increments therein. In some embodiments, the number of layers is chosen to result in an engineered meat product with thickness that approximates that of a traditional meat product, for example, a strip of bacon, a hamburger patty, a fish fillet, a chicken breast, or a steak.

[0055] In some embodiments, the engineered layers are designed to resemble traditional meat products and design parameters (e.g., cell types, additives, size, shape, etc.) are chosen to approximate those found in traditional meat products. In further embodiments, a layer is characterized by a nutritional composition that is substantially similar to traditional meat products. In still further embodiments, a layer is characterized by a nutritional composition that is substantially 60-80 percent aqueous fluid, 14-35 percent protein, 1-25 percent fat, 1-5 percent carbohydrates and 1-5 percent other substances. In some embodiments, myocytes of the engineered layers or endothelialized meat are aligned. In some embodiments, myocytes are aligned by application of an electrical field as is known in the art. In some embodiments, myocytes are aligned by application of a mechanical stimulus, such as cyclical stretching and relaxing the substratum, as is known in the art. In further embodiments, aligned (e.g., electro-oriented and mechano-oriented) myocytes have substantially the same orientation with regard to each other as is found in many animal muscle tissues. In some embodiments, layers of multicellular bodies provided herein are exposed to electrical and/or mechanical stimulation to facilitate the formation of physiological arrangement of muscle cells.

Additives

[0056] In some embodiments, the engineered meat products, engineered layers, and/or multicellular bodies include one or more nutritional supplements. In further embodiments, one or more nutritional supplements are selected from: vitamins, minerals, fiber, fatty acids, and amino acids. In some embodiments, the engineered meat products, layers, and/or multicellular bodies include one or more additives to enhance the commercial appeal (e.g., appearance, taste, color, odor, etc.). In further embodiments, the engineered meat products, layers, and/or multicellular bodies include one or more flavorants, one or more colorants, and/or one or more odorants.

[0057] In some embodiments, the engineered meat products, engineered layers, and/or multicellular bodies include one or more of: matrix proteins, proteoglycans, antioxidants, perfluorocarbons, and growth factors. The term "growth factor," as used herein, refers to a protein, a polypeptide, or a complex of polypeptides, including cytokines, that are produced by a cell and which can affect itself and/or a variety of other neighboring or distant cells. Typically growth factors affect the growth and/or differentiation of specific types of cells, either developmentally or in response to a multitude of physiological or environmental stimuli. Some, but not all, growth factors are hormones. Exemplary growth factors are insulin, insulin-like growth factor (IGF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), fibroblast growth factors (FGFs), including basic FGF (bFGF), platelet-derived growth factors (PDGFs), including PDGF-AA and PDGF-AB, hepatocyte growth factor (HGF), transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β), including TGFβ1 and TGFβ3, epidermal growth factor (EGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), interleukin-6 (IL-6), IL-8, and the like.

[0058] In some embodiments, the engineered meat products, engineered layers, and/or multicellular bodies include one or more food preservatives known to the art. In some embodiments, the preservatives are antimicrobial preservatives including, by way of non-limiting examples, calcium propionate, sodium nitrate, sodium nitrite, sulfites (e.g., sulfur dioxide, sodium bisulfite, potassium hydrogen sulfite, etc.) and disodium ethylenediaminetetraacetic acid (EDTA). In some embodiments, the preservatives are antioxidant preservatives including, by way of non-limiting examples, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT).

Support substrate

[0059] Disclosed herein, in some embodiments, is a plurality of multicellular bodies arranged adjacently on a support substrate to form a substantially planar layer for use in formation of engineered comestible meat. Also disclosed herein, in some embodiments, are methods comprising arranging multicellular bodies adjacently on a support substrate to form substantially planar layers, laying more than one layer adjacently onto a single support substrate, and allowing the layers to fuse to form engineered meat. For example, a plurality of layers may be formed as described above at the same time on different substrates then removed from their substrate when the multicellular bodies have fused sufficiently to allow them to be removed and stacked atop one another or atop a single substrate.

[0060] In general, each layer includes non-human myocytes. The cells in the central portions of such constructs are typically supplied with oxygen and nutrients by diffusion; however, gasses and nutrients typically diffuse approximately 200-300 microns into three-dimensional cellular constructs.

[0061] In some embodiments, the multicellular bodies disclosed herein have a diameter adapted to allow diffusion to sufficiently support the maintenance and growth of said non-human myocytes in culture. As a result, in

further embodiments, the layers disclosed herein have a thickness adapted to allow diffusion to sufficiently support the maintenance and growth of said non-human myocytes in culture.

[0062] To facilitate and enhance diffusion, in some embodiments, a support substrate is permeable to fluids, gasses, and nutrients and allows cell culture media to contact all surfaces of multicellular bodies and/or layers during, for example, growth, maturation, and fusion. In various embodiments, a support substrate is made from natural biomaterials, synthetic biomaterials, and combinations thereof. In some embodiments, natural biomaterials include, by way of non-limiting examples, collagen, fibronectin, laminin, and other extracellular matrices. In some embodiments, synthetic biomaterials may include, by way of non-limiting examples, hydroxyapatite, alginate, agarose, polyglycolic acid, polylactic acid, and their copolymers. In some embodiments, a support substrate is solid. In some embodiments, a support substrate is semisolid. In further embodiments, a support substrate is a combination of solid and semisolid support elements.

[0063] In some embodiments, the support substrate is raised or elevated above a non-permeable surface, such as a portion of a cell culture environment (e.g., a Petri dish, a cell culture flask, etc.) or a bioreactor. In still further embodiments, an elevated support substrate further facilitates circulation of cell culture media and enhances contact with all surfaces of the multicellular bodies and/or layers.

Methods of forming multicellular bodies

[0064] There are various ways to make multicellular bodies having the characteristics described herein. In some embodiments, a multicellular body can be fabricated from a cell paste containing a plurality of living cells or with a desired cell density and viscosity. In further embodiments, the cell paste can be shaped into a desired shape and a multicellular body formed through maturation (e.g., incubation). In a particular embodiment, a multicellular body is produced by shaping a cell paste including a plurality of living cells into a desired shape (e.g., a cylinder, a sphere). In further embodiments, the cell paste is incubated in a controlled environment to allow the cells to adhere and/or cohere to one another to form the multicellular body. In another particular embodiment, a multicellular body is produced by shaping a cell paste including a plurality of living cells in a device that holds the cell paste in a three-dimensional shape. In further embodiments, the cell paste is incubated in a controlled environment while it is held in the three dimensional shape for a sufficient time to produce a body that has sufficient cohesion to support itself on a flat surface, as described herein.

[0065] In various embodiments, a cell paste is provided by: (A) mixing cells or cell aggregates (of one or more cell types) and a cell culture medium (e.g., in a pre-determined ratio) to result in a cell suspension, and (B) compacting the cellular suspension to produce a cell paste with a desired cell density and viscosity. In various embodiments, compacting is achieved by a number of methods, such as by concentrating a particular cell suspension that resulted from cell culture to achieve the desired cell concentration (density), viscosity, and consistency required for the cell paste. In a particular embodiment, a relatively dilute cell suspension from cell culture is centrifuged for a determined time to achieve a cell concentration in the pellet that allows shaping in a mold. Tangential flow filtration ("TFF") is another suitable method of concentrating or compacting the cells. In some embodiments, compounds are combined with the cell suspension to lend the extrusion properties required. Suitable compounds include, by way of non-limiting examples, collagen, hydrogels, Matrigel, nanofibers, self-assembling nanofibers, gelatin, fibrinogen, etc.

[0066] In some embodiments, the cell paste is produced by mixing a plurality of living cells with a tissue culture medium, and compacting the living cells (e.g., by centrifugation). One or more ECM component (or derivative of an ECM component) is optionally included by resuspending the cell pellet in one or more physiologically acceptable buffers containing the ECM component(s) (or derivative(s) of ECM component(s)) and the resulting cell suspension centrifuged again to form the cell paste.

[0067] In some embodiments, the cell density of the cell paste desired for further processing may vary with cell types. In further embodiments, interactions between cells determine the properties of the cell paste, and different cell types will have a different relationship between cell density and cell-cell interaction. In still further embodiments, the cells may be pre-treated to increase cellular interactions before shaping the cell paste. For example, cells may be incubated inside a centrifuge tube after centrifugation in order to enhance cell-cell interactions prior to shaping the cell paste.

[0068] In various embodiments, many methods are used to shape the cell paste. For example, in a particular embodiment, the cell paste is manually molded or pressed (e.g., after concentration/compaction) to achieve a desired shape. By way of a further example, the cell paste may be taken up (e.g., aspirated) into a preformed instrument, such as a micropipette (e.g., a capillary pipette), that shapes the cell paste to conform to an interior surface of the instrument. The cross sectional shape of the micropipette (e.g., capillary pipette) is alternatively circular, square, rectangular, triangular, or other non-circular cross-sectional shape. In some embodiments, the cell paste is shaped by depositing it into a preformed mold, such as a plastic mold, metal mold, or a gel mold. In some embodiments, centrifugal casting or continuous casting is used to shape the cell paste.

[0069] Referring to **Fig. 5,** in a particular example, the shaping includes retaining the cell paste **4** in a shaping device **5** (e.g., a capillary pipette) to allow the cells to partially adhere and/or cohere to one another in the shap-

ing device. By way of further example, cell paste can be aspirated into a shaping device and held in the shaping device for a maturation period (also referred to herein as an incubation period) to allow the cells to at least partially adhere and/or cohere to one another. In some embodiments, the shaping device (e.g., capillary pipette) is part of a printing head of a bioprinter or similar apparatus operable to automatically place the multicellular body in a three-dimensional construct. However, there is a limit to the amount of time cells can remain in a shaping device such as a capillary pipette, which provides the cells only limited access at best to oxygen and/or nutrients, before viability of the cells is impacted.

[0070] In some embodiments, a partially adhered and/or cohered cell paste is transferred from the shaping device (e.g., capillary pipette) to a second shaping device (e.g., a mold) that allows nutrients and/or oxygen to be supplied to the cells while they are retained in the second shaping device for an additional maturation period. One example of a suitable shaping device that allows the cells to be supplied with nutrients and oxygen is a mold for producing a plurality of multicellular bodies (e.g., substantially identical multicellular bodies). By way of further example, such a mold includes a biocompatible substrate made of a material that is resistant to migration and ingrowth of cells into the substrate and resistant to adherence of cells to the substrate. In various embodiments, the substrate can suitably be made of Teflon®, (PTFE), stainless steel, agarose, polyethylene glycol, glass, metal, plastic, or gel materials (e.g., agarose gel or other hydrogel), and similar materials. In some embodiments, the mold is also suitably configured to allow supplying tissue culture media to the cell paste (e.g., by dispensing tissue culture media onto the top of the mold).

[0071] In a particular embodiment, a plurality of elongate grooves is formed in the substrate. In a further particular embodiment, the depth of each groove is in the range of about 500 microns to about 1000 microns and the bottom of each groove has a semicircular cross-sectional shape for forming elongate cylindrical multicellular bodies that have a substantially circular cross-sectional shape. In a further particular embodiment, the width of the grooves is suitably slightly larger than the width of the multicellular body to be produced in the mold. For example, the width of the grooves is suitably in the range of about 300 microns to about 1000 microns.

[0072] Thus, in embodiments where a second shaping device is used, the partially adhered and/or cohered cell paste is transferred from the first shaping device (e.g., a capillary pipette) to the second shaping device (e.g., a mold). In further embodiments, the partially adhered and/or cohered cell paste can be transferred by the first shaping device (e.g., the capillary pipette) into the grooves of a mold. In still further embodiments, following a maturation period in which the mold is incubated along with the cell paste retained therein in a controlled environment to allow the cells in the cell paste to further adhere and/or cohere to one another to form the multicellular body, the cohesion of the cells will be sufficiently strong to allow the resulting multicellular body to be picked up with an implement (e.g., a capillary pipette). In still further embodiments, the capillary pipette is suitably be part of a printing head of a bioprinter or similar apparatus operable to automatically place the multicellular body into a three-dimensional construct.

[0073] In some embodiments, the cross-sectional shape and size of the multicellular bodies will substantially correspond to the cross-sectional shapes and sizes of the first shaping device and optionally the second shaping device used to make the multicellular bodies, and the skilled artisan will be able to select suitable shaping devices having suitable cross-sectional shapes, cross-sectional areas, diameters, and lengths suitable for creating multicellular bodies having the cross-sectional shapes, cross-sectional areas, diameters, and lengths discussed above.

[0074] As discussed herein, a large variety of cell types may be used to create the multicellular bodies of the present embodiments. Thus, one or more types of cells or cell aggregates including, for example, all of the cell types listed herein, may be employed as the starting materials to create the cell paste. For instance, cells such as non-human myocytes, endothelial cells, adipose cells, and fibroblasts are optionally employed. As described herein, a multicellular body is homocellular or heterocellular. For making homocellular multicellular bodies, the cell paste suitably is homocellular, i.e., it includes a plurality of living cells of a single cell type. For making heterocellular multicellular bodies, on the other hand, the cell paste will suitably include significant numbers of cells of more than one cell type (i.e., the cell paste will be heterocellular). As described herein, when heterocellular cell paste is used to create the multicellular bodies, the living cells may "sort out" during the maturation and cohesion process based on differences in the adhesive strengths of the cells, and may recover their physiological conformation.

[0075] In some embodiments, in addition to the plurality of living cells, one or more ECM components or one or more derivatives of one or more ECM components (e.g., gelatin, fibrinogen, collagen, fibronectin, laminin, elastin, and/or proteoglycans) can suitably be included in the cell paste to incorporate these substances into the multicellular bodies, as noted herein. In further embodiments, adding ECM components or derivatives of ECM components to the cell paste may promote cohesion of the cells in the multicellular body. For example, gelatin and/or fibrinogen are optionally added to the cell paste. More particularly, a solution of 10-30% gelatin and a solution of 10-80 mg/ml fibrinogen are optionally mixed with a plurality of living cells to form a cell suspension containing gelatin and fibrinogen.

[0076] Various methods are suitable to facilitate the further maturation process. In one embodiment, the cell paste may be incubated at about 37°C for a time period (which may be cell-type dependent) to foster adherence

and/or coherence. Alternatively or in addition, the cell paste may be held in the presence of cell culture medium containing factors and/or ions to foster adherence and/or coherence.

Arranging multicellular bodies on a support substrate to form layers

[0077] A number of methods are suitable to arrange multicellular bodies on a support substrate to produce a desired three-dimensional structure (e.g., a substantially planar layer). For example, in some embodiments, the multicellular bodies are manually placed in contact with one another, deposited in place by extrusion from a pipette, nozzle, or needle, or positioned in contact by an automated machine such as a bioprinter.

[0078] As described herein, in some embodiments, the support substrate is permeable to fluids, gasses, and nutrients and allows cell culture media to contact all surfaces of the multicellular bodies and/or layers during arrangement and subsequent fusion. As further described herein, in some embodiments, a support substrate is made from natural biomaterials such as collagen, fibronectin, laminin, and other extracellular matrices. In some embodiments, a support substrate is made from synthetic biomaterials such as hydroxyapatite, alginate, agarose, polyglycolic acid, polylactic acid, and their copolymers. In some embodiments, a support substrate is solid. In some embodiments, a support substrate is semisolid. In further embodiments, a support substrate is a combination of solid and semisolid support elements. In further embodiments, a support substrate is planar to facilitate production of planar layers. In some embodiments, the support substrate is raised or elevated above a non-permeable surface, such as a portion of a cell culture environment (e.g., a Petri dish, a cell culture flask, etc.) or a bioreactor. Therefore, in some embodiments, a permeable, elevated support substrate contributes to prevention of premature cell death, contributes to enhancement of cell growth, and facilitates fusion of multicellular bodies to form layers.

[0079] As described herein, in various embodiments, multicellular bodies have many shapes and sizes. In some embodiments, multicellular bodies are elongate and in the shape of a cylinder. *See* e.g., **Figs. 1** and **3.** In some embodiments, multicellular bodies provided herein are of similar lengths and/or diameters. In other embodiments, multicellular bodies provided herein are of differing lengths and/or diameters. In some embodiments, multicellular bodies are substantially spherical. *See* e.g., **Figs. 2** and **4.** In some embodiments, layers include substantially spherical multicellular bodies that are substantially similar in size. In other embodiments, layers include substantially spherical multicellular bodies that are of differing sizes.

[0080] Referring to **Fig. 6,** in some embodiments, multicellular bodies **1** are arranged on a support substrate **3** horizontally adjacent to, and in contact with, one or more other multicellular bodies to form a substantially planar layer.

[0081] Referring to **Fig. 7,** in some embodiments, substantially spherical multicellular bodies **2** are arranged on a support substrate **3** horizontally adjacent to, and in contact with, one or more other substantially spherical multicellular bodies. In further embodiments, this process is repeated to build up a pattern of substantially spherical multicellular bodies, such as a grid, to form a substantially planar layer.

[0082] Referring to **Fig. 8,** in a particular embodiment, a multicellular **6** body is laid onto a support substrate **3** via an implement such as a capillary pipette **5** such that it is horizontally adjacent to, and in contact with one or more other multicellular bodies. In further embodiments, a multicellular body is laid onto a support substrate such that it is parallel with a plurality of other multicellular bodies.

[0083] Referring to **Fig. 9,** in some embodiments, a subsequent series of multicellular bodies **8** are arranged vertically adjacent to, and in contact with, a prior series of multicellular bodies **9** on a support substrate **3** to form a thicker layer.

[0084] In other embodiments, layers of different shapes and sizes are formed by arranging multicellular bodies of various shapes and sizes. In some embodiments, multicellular bodies of various shapes, sizes, densities, cellular compositions, and/or additive compositions are combined in a layer and contribute to, for example, appearance, taste, and texture of the resulting layer.

[0085] Referring to **Fig. 10,** in some embodiments, elongate multicellular bodies **9** are arranged adjacent to, and in contact with, substantially spherical multicellular bodies **10** on a support substrate **3** to form a complex layer.

[0086] Once assembly of a layer is complete, in some embodiments, a tissue culture medium is poured over the top of the construct. In further embodiments, the tissue culture medium enters the spaces between the multicellular bodies to support the cells in the multicellular bodies. The multicellular bodies in the three-dimensional construct are allowed to fuse to one another to produce a substantially planar layer for use in formation of engineered, comestible meat. By "fuse," "fused" or "fusion," it is meant that the cells of contiguous multicellular bodies become adhered and/or cohered to one another, either directly through interactions between cell surface proteins, or indirectly through interactions of the cells with extracellular matrix (ECM) components or derivatives of ECM components. In some embodiments, the cells within the multicellular bodies produce their own cell specific ECM (e.g., collagen), which provides the mechanical integrity of the multicellular bodies and the comestible meat product. In some embodiments, a fused layer is completely fused and that multicellular bodies have become substantially contiguous. In some embodiments, a fused layer is substantially fused or partially fused and the cells

of the multicellular bodies have become adhered and/or cohered to the extent necessary to allow moving and manipulating the layer intact.

[0087] In some embodiments, the multicellular bodies fuse to form a layer in a cell culture environment (e.g., a Petri dish, cell culture flask, bioreactor, etc.). In further embodiments, the multicellular bodies fuse to form a layer in an environment with conditions suitable to facilitate growth of the cell types included in the multicellular bodies. In various embodiments, fusing takes place over about 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 minutes, and increments therein. In other various embodiments, fusing takes place over about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, and 48 hours, and increments therein. In yet other various embodiments, fusing takes place over about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, and 14 days, and increments therein. In further embodiments, fusing takes place over about 2 hours to about 36 hours. Several factors influence the fusing time required including, by way of non-limiting examples, cell types, cell type ratios, culture conditions, and the presence of additives such as growth factors.

[0088] Once fusion of a layer is complete, in some embodiments, the layer and the support substrate are separated. In other embodiments, the layer and the support substrate are separated when fusion of a layer is substantially complete or partially complete, but the cells of the layer are adhered and/or cohered to one another to the extent necessary to allow moving, manipulating, and stacking the layer without breaking it apart. In further embodiments, the layer and the support substrate are separated via standard procedures for melting, dissolving, or degrading the support substrate. In still further embodiments, the support substrate is dissolved, for example, by temperature change, light, or other stimuli that do not adversely affect the layer. In a particular embodiment, the support substrate is made of a flexible material and peeled away from the layer.

[0089] In some embodiments, the separated layer is transferred to a bioreactor for further maturation. In some embodiments, the separated layer matures and further fuses after incorporation into an engineered meat product.

[0090] In other embodiments, the layer and the support substrate are not separated. In further embodiments, the support substrate degrades or biodegrades prior to packaging, freezing, sale or consumption of the assembled engineered meat product.

Arranging layers on a support substrate to form engineered meat

[0091] A number of methods are suitable to arrange layers on a support substrate to produce engineered meat. For example, in some embodiments, the layers are manually placed in contact with one another or deposited in place by an automated, computer-aided machine such as a bioprinter, according to a computer script. In further embodiments, substantially planar layers are stacked to form engineered meat.

[0092] In various embodiments, about 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, or 500 layers, or increments therein, are stacked. In further embodiments, stacking is repeated to develop a thickness that approximates a traditional meat product such as a Carpaccio, a strip of bacon, a hamburger patty, a fish fillet, a chicken breast, or a steak. In various embodiments, stacked layers comprise an engineered meat product about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 mm, or increments therein, thick.

[0093] In some embodiments, a layer has an orientation defined by the placement, pattern, or orientation of multicellular bodies. In further embodiments, each layer is stacked with a particular orientation relative to the support substrate and/or one or more other layers. In various embodiments, one or more layers is stacked with an orientation that includes rotation relative to the support substrate and/or the layer below, wherein the rotation is about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, and 180 degrees, or increments therein. In other embodiments, all layers are oriented substantially similarly.

[0094] Referring to **Fig. 11,** in a particular embodiment, layers have an orientation defined by the parallel placement of multicellular bodies used to form the layer. In a further particular embodiment, layers are stacked with an orientation including 90 degree rotation with respect to the layer below to form engineered meat.

[0095] Once stacking of the layers is complete, in some embodiments, the layers in the three-dimensional construct are allowed to fuse to one another to produce engineered meat. In some embodiments, the layers fuse to form engineered meat in a cell culture environment (e.g., a Petri dish, cell culture flask, bioreactor, etc.). In various embodiments, fusing takes place over about 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 minutes, and increments therein. In other various embodiments, fusing takes place over about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, and 48 hours, and increments therein. In further embodiments, fusing takes place over about 2 hours to about 36 hours.

[0096] In some embodiments, once stacked, the cells of the multicellular bodies and layers begin to die due to the inability of gases, fluids, and nutrients, to diffuse into or otherwise reach the inner portions of the construct. In further embodiments, the gradual death of the cells is similar to the natural cell death that occurs in the tissues of a postmortem organism. In some embodiments, the layers of the engineered meat construct fuse to one another simultaneously with the gradual death of the cells. In some embodiments, the multicellular bodies of the layers continue to fuse to one another simultaneously with the gradual death of the cells. In further embodiments,

fusion within and between layers is complete or substantially complete prior to the death of a majority of the cells of the construct. In further embodiments, fusion within and between layers is complete or substantially complete prior to the death of all the cells of the construct.

[0097] Once assembly of the engineered meat is complete, in some embodiments, the meat and the support substrate are separated. In further embodiments, the meat and the support substrate are separated via standard procedures for melting, dissolving, or degrading the support substrate. In still further embodiments, the support substrate is dissolved, for example, by temperature change, light, or other stimuli that do not adversely affect the meat. In a particular embodiment, the support substrate is made of a flexible material and peeled away from the meat. In some embodiments, the separated meat is transferred to a bioreactor for further maturation. In other embodiments, the meat and the support substrate are not separated. In further embodiments, the support substrate degrades or biodegrades prior to sale or consumption.

[0098] In some embodiments, the meat is irradiated. In some embodiments, the meat is frozen to prevent decomposition or degradation prior to distribution, sale, and consumption. In further embodiments, frozen meat is vacuum-packed.

Engineered meat

[0099] Disclosed herein, in some embodiments, are engineered meat products obtained according to the methods of the invention.

[0100] In some embodiments, the engineered meat products are fresh. In other embodiments, the engineered meat products are preserved. In further embodiments, the meat is preserved by, for example, cooking, drying, smoking, canning, pickling, salt-curing, or freezing.

[0101] In some embodiments, the engineered meat products are substantially-free of pathogenic microorganisms. In further embodiments, controlled and substantially sterile methods of cell preparation, cell culture, multicellular body preparation, layer preparation, and engineered meat preparation result in a product substantially-free of pathogenic microorganisms. In further embodiments, an additional advantage of such a product is increased utility and safety.

[0102] In some embodiments, the engineered meat products are shaped. In further embodiments, the meat is shaped by, for example, controlling the number, size, and arrangement of the multicellular bodies and/or the layers used to construct the meat. In other embodiments, the meat is shaped by, for example, cutting, pressing, molding, or stamping. In some embodiments, the shape of a meat product is selected to resemble a traditional meat product such as a strip of bacon, a sausage link, a sausage patty, a hamburger patty, a hot dog, a fish fillet, a chicken breast, a chicken strip, a chicken nugget, a meatloaf, or a steak. In other embodiments, the engineered meat products are ground.

EXAMPLES

[0103] The following illustrative examples are representative of embodiments described herein and are not meant to be limiting in any way.

Example 1 - Preparation of support substrate

[0104] To prepare a 2% agarose solution, 2 g of Ultrapure Low Melting Point (LMP) agarose was dissolved in 100 mL of ultrapure water/buffer solution (1:1, v/v). The buffer solution is optionally PBS (Dulbecco's phosphate buffered saline 1x) or HBSS (Hanks' balanced salt solution 1x). The agarose solution was placed in a beaker containing warm water (over 80°C) and held on the hot plate until the agarose dissolves completely. The agarose solution remains liquid as long as the temperature is above 36°C. Below 36°C, a phase transition occurs, the viscosity increases, and finally the agarose forms a gel.

[0105] To prepare agarose support substrate, 10 mL of liquid 2% agarose (temperature >40°C) was deposited in a 10 cm diameter Petri dish and evenly spread to form a uniform layer. Agarose was allowed to form a gel at 4°C in a refrigerator.

Example 2 - Culture of porcine aortic smooth muscle cells

[0106] Freshly isolated porcine aortic smooth muscle cells (PASMCs) were grown in low glucose DMEM with 10% fetal bovine serum (Hyclone Laboratories, UT), 10% porcine serum (Invitrogen), L-ascorbic acid, copper sulfate, HEPES, L-proline, L-alanine, L-glycine, and Penicillin G (all aforementioned supplements were purchased from Sigma, St. Louis, MO). Cell lines were cultured on 0.5% gelatin (porcine skin gelatin; Sigma) coated dishes (Techno Plastic Products, St. Louis, MO) and were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The PASMCs were subcultured up to passage 7 before being used to form multicellular bodies.

Example 3 - Preparation of multicellular spheroids and cylinders

[0107] Cell cultures were washed twice with phosphate buffered saline solution (PBS, Invitrogen) and treated for 10 min with 0.1% Trypsin (Invitrogen) and centrifuged at 1500 RPM for 5 min. Cells were resuspended in 4 mL of cell-type specific medium and incubated in 10-mL tissue culture flasks (Bellco Glass, Vineland, NJ) at 37°C with 5% CO_2 on gyratory shaker (New Brunswick Scientific, Edison, NJ) for one hour, for adhesion recovery and centrifuged at 3500 RPM. The resulting pellets were transferred into capillary micropipettes of 300 μm (Sutter In-

strument, CA) or 500 μm (Drummond Scientific Company, Broomall, PA) diameters and incubated at 37°C with 5% CO_2 for 15 min. For spherical multicellular bodies, extruded cylinders were cut into equal fragments that were let to round up overnight on a gyratory shaker. Depending on the diameter of the micropipettes, this procedure provided regular spheroids of defined size and cell number. For cylindrical multicellular bodies, cylinders were mechanically extruded into specifically prepared non-adhesive Teflon® or agarose molds using a bioprinter. After overnight maturation in the mold, cellular cylinders were cohesive enough to be deposited.

[0108] The multicellular bodies were packaged into cartridges (micropipettes of 300-500 μm inner diameter). Cartridges were inserted into a bioprinter and delivered onto a support substrate according to a computer script that encodes the shape of the structure to be printed.

Example 4 - Preparation of engineered meat

[0109] Cylindrical multicellular bodies are prepared as described in **Example 3.** The multicellular bodies are heterocellular and composed of the PASMCs of **Example 2** and Porcine Coronary Artery Endothelial Cells (PCAEC, Genlantis, San Diego, CA, Product No. PP30005). The ratio of myocytes to endothelial cells in the multicellular bodies is about 6:1. The multicellular bodies have a cross-sectional diameter of 300 μm and a length of either 2 cm, 3 cm, 4 cm, or 5 cm. Matured and multicellular bodies are packaged into cartridges (micropipettes of 300 μm inner diameter), which are then inserted into a bioprinter.

[0110] An agarose support substrate is prepared as described in **Example 1.** The support substrate is raised above the bottom of a large Petri dish by a fine mesh pedestal such that cell culture media may contact all surfaces of the multicellular bodies and layers deposited onto the substrate.

[0111] A bioprinter delivers the multicellular bodies onto the support substrate according to the instructions of a computer script. The script encodes placement of cylindrical multicellular bodies to form a substantially square monolayer with an average width of about 10 cm and an average length of about 10 cm. The multicellular bodies are placed parallel to one another with bodies of varying lengths placed end to end to form the encoded shape.

[0112] Culture medium is poured over the top of the layer and the construct is allowed to partially fuse over the course of about 12 hours at 37°C in a humidified atmosphere containing 5% CO_2. During this time, the cells of the multicellular bodies adhere and/or cohere to the extent necessary to allow moving and manipulating the layer without breaking it apart.

[0113] The partially fused layers are peeled from the support and stacked. Sixty-five layers are stacked to form the engineered meat, which has an overall width and height of about 2 cm and a length and width of about 10 cm. Each layer is rotated 90 degrees with respect to the layer below. Once stacked, the cells start dying due to oxygen deprivation, as culture medium is not changed. Cell death starts in the stack's interior, as these are the first deprived of oxygen, and progressively reaches outer cells, as the surrounding culture medium gets gradually depleted in oxygen. Simultaneously with cell death the partially fused layers continue to fuse while they start fusing also in the vertical direction. Since the fusion process takes about 6 hours, while cell death takes about 20 hours, the postmortem construct is fully fused and assumes a shape similar to a square pork hamburger patty.

Claims

1. A method of forming engineered meat, the method comprising:

   preparing a plurality of multicellular bodies comprising a plurality of non-human myocytes cohered to one another;
   laying more than one multicellular body adjacently onto a planar support substrate;
   fusing said multicellular bodies at least partially together to form a first layer;
   stacking more than 50 additional layers onto the first layer;
   fusing the stacked layers to form a volume of engineered meat; and
   culturing the stacked layers to fuse the layers while the layers in an inner region of the volume die such that the majority of cells in the volume have died after fusion between the layers is at least partially complete, and
   wherein the engineered meat is comestible.

2. The method of claim 1, further comprising freezing said volume of engineered meat.

3. The method of claim 1, wherein preparing the plurality of multicellular bodies comprises preparing a plurality of elongate multicellular bodies comprising a plurality of non-human myocytes cohered to one another and preparing a plurality of substantially spherical multicellular bodies comprising a plurality of non-human myocytes cohered to one another; and further wherein laying more than one multicellular body comprises laying more than one elongate multicellular body and more than one substantially spherical multicellular body adjacently onto a planar support substrate.

4. The method of claim 3, wherein said elongate multicellular bodies have a length ranging from 1 mm to 10 cm.

5. The method of claim 1, wherein preparing the plu-

rality of multicellular bodies comprises preparing a plurality of substantially spherical multicellular bodies comprising a plurality of non-human myocytes cohered to one another; and further wherein laying more than one multicellular body comprises laying more than one substantially spherical multicellular body adjacently onto a support substrate.

6. The method of claim 1, wherein the planar support substrate is permeable to fluids and nutrients and allows cell culture media to contact all surfaces of the multicellular bodies.

7. The method of claim 1, wherein said multicellular bodies have a diameter of 50 μm to 1000 μm to allow diffusion to sufficiently support the maintenance and growth of said non-human myocytes and non-human endothelial cells in culture.

8. The method of claim 1, wherein said multicellular bodies have a diameter of 100 μm to 500 μm.

Patentansprüche

1. Verfahren zur Bildung von konstruiertem Fleisch, wobei das Verfahren Folgendes umfasst:

Herstellen einer Vielzahl von multizellulären Körpern, die eine Vielzahl von nicht-menschlichen Myozyten umfasst, die aneinander zusammenhalten;
benachbartes Auslegen von mehr als einem multizellulären Körper auf einem ebenen Trägersubstrat;
zumindest teilweises Zusammenfusionieren der multizellulären Körper, um eine erste Schicht zu bilden;
Aufschichten von mehr als 50 zusätzlichen Schichten auf die erste Schicht:

Fusionieren der aufgeschichteten Schichten, um ein Volumen von konstruiertem Fleisch zu bilden; und
Kultivieren der aufgeschichteten Schichten, um die Schichten zu fusionieren, während die Schichten in einem inneren Bereich des Volumens absterben, sodass die Mehrheit der Zellen in dem Volumen abgestorben ist, nachdem die Fusion zwischen den Schichten zumindest teilweise vollständig ist, und wobei das konstruierte Fleisch essbar ist.

2. Verfahren nach Anspruch 1, das weiter das Gefrieren des Volumens von konstruiertem Fleisch umfasst.

3. Verfahren nach Anspruch 1, wobei das Herstellen der Vielzahl von multizellulären Körpern Folgendes umfasst: Herstellen einer Vielzahl von länglichen multizellulären Körpern, die eine Vielzahl von nicht-menschlichen Myozyten umfasst, die aneinander zusammenhalten, und Herstellen einer Vielzahl von im Wesentlichen kugelförmigen multizellulären Körpern, die eine Vielzahl von nicht-menschlichen Myozyten umfasst, die aneinander zusammenhalten; und weiter wobei das Auslegen von mehr als einem multizellulären Körper das benachbarte Auslegen von mehr als einem länglichen multizellulären Körper und von mehr als einem im Wesentlichen kugelförmigen multizellulären Körper auf einem ebenen Trägersubstrat umfasst.

4. Verfahren nach Anspruch 3, wobei die länglichen multizellulären Körper eine Länge im Bereich von 1 mm bis 10 cm aufweisen.

5. Verfahren nach Anspruch 1, wobei das Herstellen der Vielzahl von multizellulären Körpern das Herstellen einer Vielzahl von im Wesentlichen kugelförmigen multizellulären Körpern, die eine Vielzahl von nicht-menschlichen Myozyten umfasst, die aneinander zusammenhalten, umfasst; und weiter wobei das Auslegen von mehr als einem multizellulären Körper das benachbarte Auslegen von mehr als einem im Wesentlichen kugelförmigen multizellulären Körper auf einem Trägersubstrat umfasst.

6. Verfahren nach Anspruch 1, wobei das ebene Trägersubstrat für Flüssigkeiten und Nährstoffe durchlässig ist und ermöglicht, dass ein Zellkulturmedium alle Oberflächen der multizellulären Körper kontaktiert.

7. Verfahren nach Anspruch 1, wobei die multizellulären Körper einen Durchmesser von 50 μm bis 1000 μm aufweisen, um Diffusion zu ermöglichen, um die Erhaltung und das Wachstum der nicht-menschlichen Myozyten und nicht-menschlichen Endothelzellen in Kultur ausreichend zu unterstützen.

8. Verfahren nach Anspruch 1, wobei die multizellulären Körper einen Durchmesser von 100 μm bis 500 μm aufweisen.

Revendications

1. Méthode de façonnage de viande artificielle, la méthode comprenant :

la préparation d'une pluralité de corps multicellulaires comprenant une pluralité de myocytes non humains collés les uns aux autres ;
le dépôt de plus d'un corps multicellulaire de manière adjacente sur un substrat support

planaire ;
la fusion desdits corps multicellulaires au moins partiellement ensemble afin de former une première couche ;
l'empilement de plus de 50 couches supplémentaires sur la première couche ;
la fusion des couches empilées afin de former un volume de viande artificielle ; et
la culture des couches empilées afin de fusionner les couches tandis que les couches dans une région interne du volume meurent de sorte que la majorité des cellules dans le volume soient mortes après que la fusion entre les couches est au moins partiellement terminée, et
où la viande artificielle est comestible.

2. Méthode selon la revendication 1, comprenant en outre la congélation dudit volume de viande artificielle.

3. Méthode selon la revendication 1, où la préparation de la pluralité de corps multicellulaires comprend la préparation d'une pluralité de corps multicellulaires allongés comprenant une pluralité de myocytes non humains collés les uns aux autres et la préparation d'une pluralité de corps multicellulaires sensiblement sphériques comprenant une pluralité de myocytes non humains collés les uns aux autres ; et en outre où le dépôt de plus d'un corps multicellulaire comprend le dépôt de plus d'un corps multicellulaire allongé et plus d'un corps multicellulaire sensiblement sphérique de manière adjacente sur un substrat support planaire.

4. Méthode selon la revendication 3, où lesdits corps multicellulaires allongés possèdent une longueur allant de 1 mm à 10 cm.

5. Méthode selon la revendication 1, où la préparation de la pluralité de corps multicellulaires comprend la préparation d'une pluralité de corps multicellulaires sensiblement sphériques comprenant une pluralité de myocytes non humains collés les uns aux autres ; et en outre où le dépôt de plus d'un corps multicellulaire comprend le dépôt de plus d'un corps multicellulaire sensiblement sphérique de manière adjacente sur un substrat support.

6. Méthode selon la revendication 1, où le substrat support planaire est perméable aux fluides et aux nutriments et permet au milieu de culture cellulaire d'entrer en contact avec toutes les surfaces des corps multicellulaires.

7. Méthode selon la revendication 1, où lesdits corps multicellulaires possèdent un diamètre allant de 50 μm à 1000 μm pour permettre une diffusion afin de supporter de manière suffisante le maintien et la croissance desdits myocytes non humains et desdites cellules endothéliales non humaines en culture.

8. Méthode selon la revendication 1, où lesdits corps multicellulaires possèdent un diamètre allant de 100 μm à 500 μm.

Fig. 1

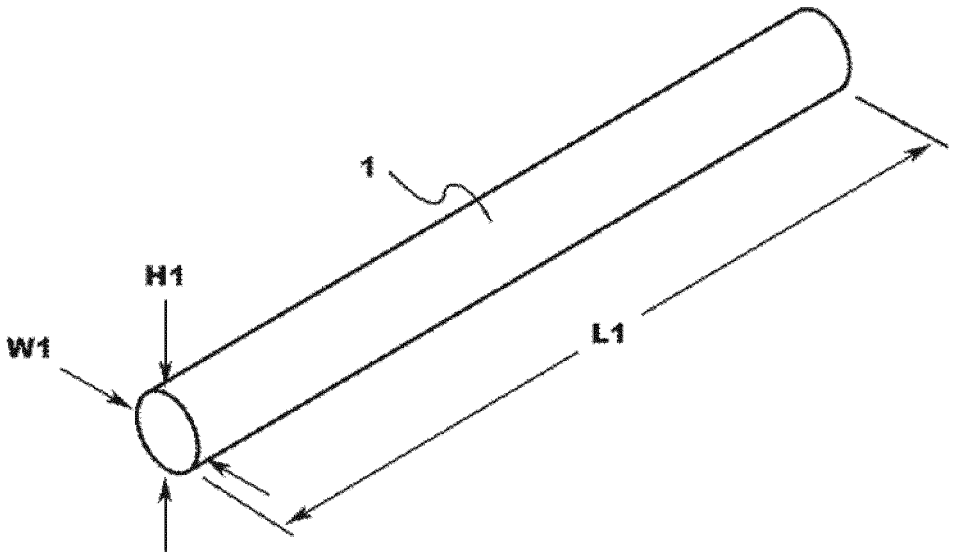


Fig. 2

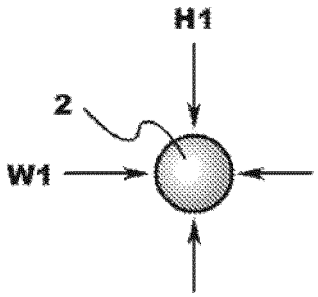


Fig. 3

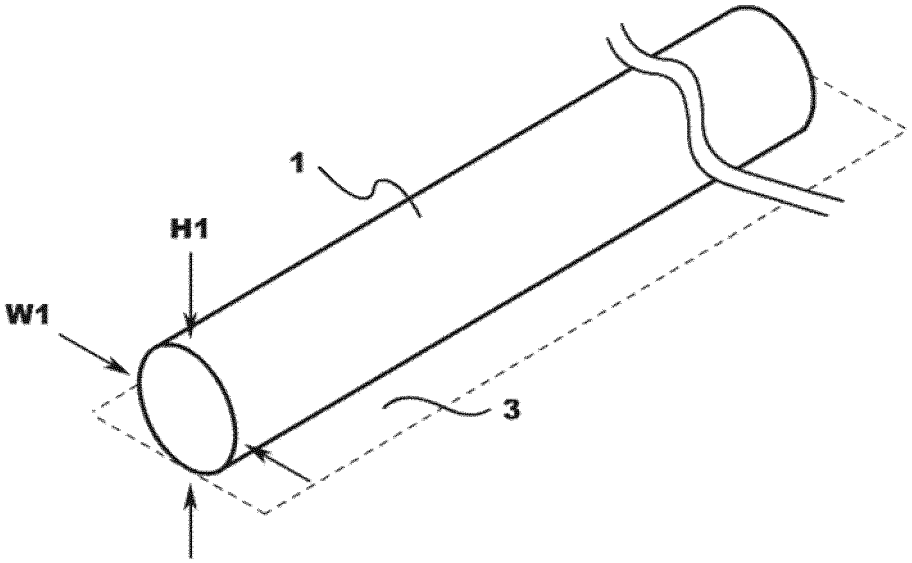


Fig. 4

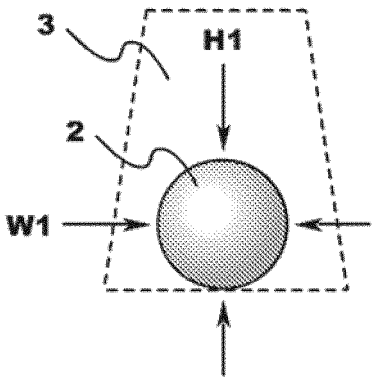


Fig. 5

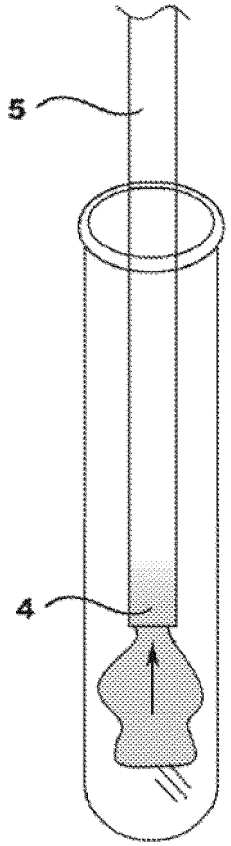


Fig. 6

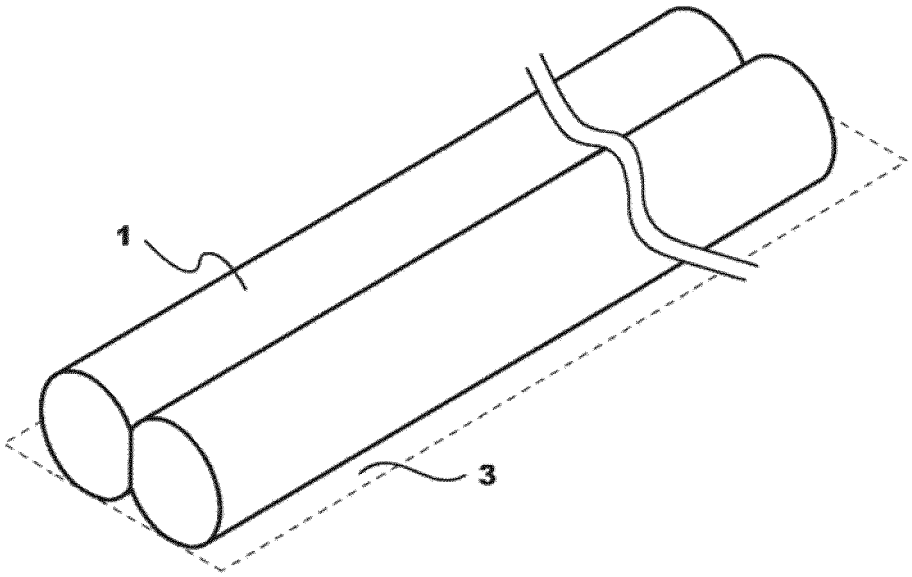


Fig. 7

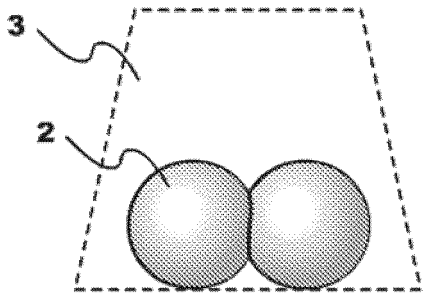


Fig. 8

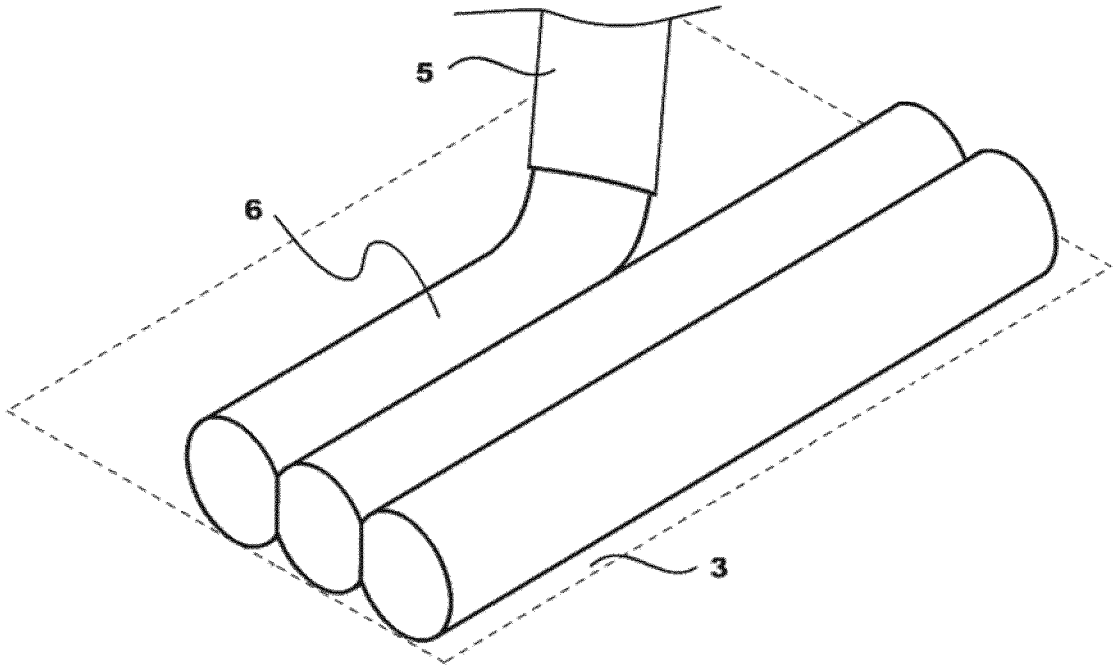


Fig. 9

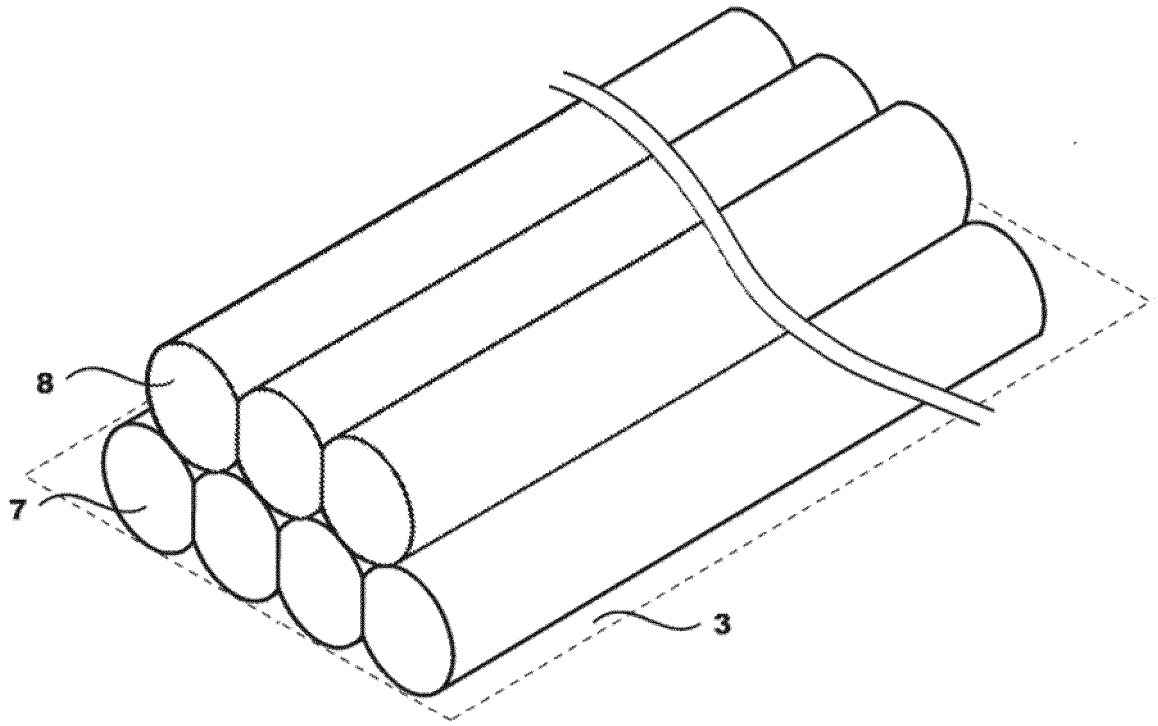


Fig. 10

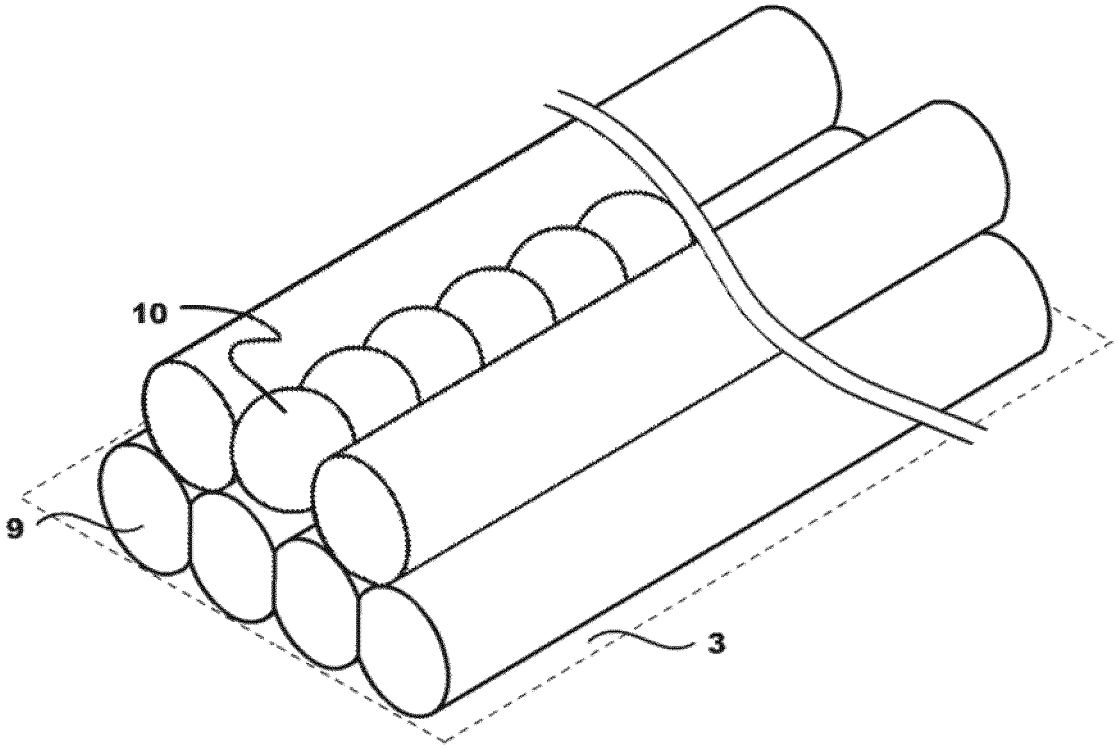
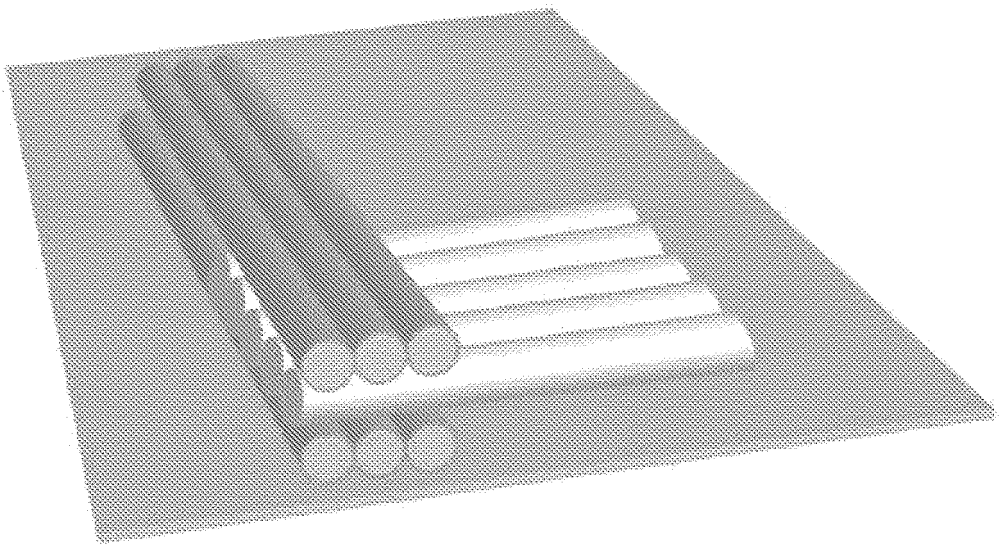


Fig. 11



REFERENCES CITED IN THE DESCRIPTION

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