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(71) Applicant: **MODERN MEADOW, INC.** [US/US]; 1601 S. Providence Road, Columbia, MO 65211-3460 (US).

(72) Inventors: **MARGA, Francoise, Suzanne**; 1601 S. Providence Road, Columbia, MO 65211-3460 (US). **FORGACS, Gabor**; 1601 S. Providence Road, Columbia, MO 65211-3460 (US).

(74) Agents: **SHOOP, Richard, D.** et al.; Shay Glenn LLP, 2755 Campus Drive, Suite 210, San Mateo, CA 94403 (US).

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(54) Title: SPHERICAL MULTICELLULAR AGGREGATES WITH ENDOGENOUS EXTRACELLULAR MATRIX

(57) Abstract: Methods of forming multicellular aggregates having cell-specific extracellular matrix (ECM), multicellular aggregates having cell-specific ECM, and methods for using these multicellular aggregates. In particular, described herein are methods of forming multicellular aggregates including forming a confluent layer of cells that is initially adhered to a substrate, removing the layer from the substrate and allowing it to ball up, and culturing the balled up layer on a non-adhesive substrate to form the multicellular aggregate.

SPHERICAL MULTICELLULAR AGGREGATES WITH ENDOGENOUS EXTRACELLULAR MATRIX

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/698,446, filed September 7, 2012 and titled "SPHERICAL MULTICELLULAR AGGREGATES WITH ENDOGENOUS EXTRACELLULAR MATRIX", which is herein incorporated by reference in its entirety.

INCORPORATION BY REFERENCE

[0002] All publications and patent applications mentioned in this specification are herein incorporated by reference in their entirety to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

FIELD

[0003] The multicellular aggregates, methods for forming them and methods of using them described herein may relate to the fields of regenerative medicine, biomaterial and tissue engineering, pharmacology (e.g., drug testing), and academic research.

BACKGROUND

[0004] Multicellular aggregates typically include groups of cells that are co-adherent. Multicellular aggregates have been used in the field of tissue engineering, however, classical tissue engineering relies on exogenous scaffolds that mimic the endogenous extracellular matrix (ECM) to culture cells in three dimensions. For example, multicellular bodies (aggregates) described in the prior art typically require a matrix ("carrier matrix") to form. The carrier or matrix structure is formed of a material compatible with the cells that is colonized with differentiated cells of the target tissue, and the cultivation of the cells in vitro until a tissue-like cell structure has been produced. The differentiated cells are obtained either from cultures of explanted tissue samples or from stem cells that had been stimulated to differentiate. The use of stem cells permits a more rapid production of larger amounts of the desired cells in many instances. Traditionally, pure cell populations of a certain type are produced. Most of the in vitro organs or in vitro tissues known in the state of the art are disadvantageous in as far as that they do not have or do not develop the tissue structure that corresponds to the morphological constitution of the native tissue or organ even after implantation and a fairly long residence time

in the body. This applies as a rule even when the carrier matrix had been colonized with several different populations of tissue-typical cells. In particular, cell aggregates formed in this way typically do not exhibit normal (endogenous) extracellular matrix (ECM).

[0005] Other examples of multicellular aggregates have been described that do not require the use of a carrier or support matrix. For example, US 8,143,055 to Forgacs et al. describes multicellular bodies that can be cultured and used to form three-dimensional constructs. However, these aggregates typically form a small amount of ECM.

[0006] The invention described herein provides a method for scaffold free tissue engineering which relies on only cells and cell-produced components, in particular on cell specific ECM produced by the cells in the aggregate. For example, described herein are multicellular aggregates with natural ECM incorporated in them and methods of preparing these aggregates. No such methods have been described earlier. Using these aggregates to build planar tissue constructs represents an efficient, rapid and cheap, scaffold-free tissue engineering method. The aggregates can be used to build more complex structures and are not limited to sheets, such as tubes.

[0007] Thus, described herein are methods of forming multicellular aggregates with cell-specific extracellular matrix, multicellular aggregates formed in this manner, and their application to engineer biomaterials. In contrast to prior art methods and aggregates, the aggregates described herein are spherical aggregates having a substantial amount of cell-specific (e.g., endogenous) ECM.

SUMMARY OF THE DISCLOSURE

[0008] Described herein are methods of forming multicellular aggregates having cell-specific ECM, multicellular aggregates having cell-specific ECM, and methods for using these multicellular aggregates.

[0009] For example, described herein are substantially spherical multicellular aggregates containing ECM produced by the cells forming the aggregate, as well as methods of forming these substantially spherical multicellular aggregates. In general these spherical aggregates may self-assemble as part of a two-step process; cells may initially be cultured to confluence in a layer, then the confluent layer may be removed from a substrate and allowed to round up (curl up or ball up) to form spherical structures, and the balled-up layer may be again cultured to form the multicellular aggregate.

[00010] The multicellular aggregates formed as described herein contain a significant amount of self-produced extracellular matrix (ECM). The ECM is typically appropriate for the cells forming the multicellular aggregates (e.g., fibrillar collagen). In some variations, ECM formation

may be induced. For example, collagen synthesis in the multicellular aggregates may be induced by addition of ascorbic acid to the initial cell solution.

[00011] This multicellular aggregates and methods of forming multicellular aggregates described herein differs from previous described multicellular aggregates and method used for preparing them. For example, the aggregates may be considered somewhat "loose," as the cells do not need to be centrifuged, and the amount of cell-specific ECM is much higher than previously reported. Other differences are highlighted and described below.

[00012] Once formed, the spherical multicellular aggregates may be used to form engineered biomaterials, including engineered tissues. For example, the multicellular aggregates described herein can be assembled into layers (by placing the aggregates contiguously to each other) on top of a non-adherent surface and fused. Thus, thick multicellular sheets (e.g., model tissue) may result from the fusion of the aggregates. The versatility of the method and the aggregates formed may provide a broad applicability in the basic sciences, pharmacology, tissue engineering and regenerative medicine fields. For example, the methods for forming multicellular aggregates described herein may provide a better model for 3D tissues than previously known, as the collagen content/structure of these multicellular aggregates described herein mimics physiological organization. Second, these aggregates may be used to form extended three-dimensional cellular/tissue structures that could be used in the food industry (e.g., to form engineered meat), or biomaterials industry (e.g., to form leather).

BRIEF DESCRIPTION OF THE DRAWINGS

[00013] FIG. 1 shows a multilayered sheet of human aortic smooth muscle cells detaching from a well after 10 days of culture with ascorbic acid (50ug/ml).

[00014] FIG. 2A shows a layer or sheet of cells detaching from a well after 10 days in ascorbic acid; FIG. 2B shows the layer completely peeled off of the well and placed on top of an agarose bed in supplemental medium.

[00015] FIG. 3 shows a substantially spherical multicellular aggregate of bovine fibroblast cells, containing intrinsic ECM, formed as described herein.

[00016] FIG. 4 illustrates one example of a method of releasing a layer/sheet of cells from an adherent substrate so that they may be placed onto a non-adherent substrate and allowed to ball up.

[00017] FIGS. 5A and 5B show a section of an aggregate composed of human aortic smooth muscle cells. FIG. 5A shows H&E staining; FIG. 5B shows Picrosirius red. In FIG. 5B, Fibrillar collagen appears red.

[00018] FIG. 6 illustrates fusion of two substantially spherical multicellular aggregates as described herein. In this example, the aggregates are human aortic smooth muscle cell aggregates.

DETAILED DESCRIPTION

[00019] In general, a multicellular aggregate (which may be referred to herein as a multicellular body) includes a plurality of living cells that are cohered to one another. A spherical multicellular aggregate typically comprises a plurality of cells cohered together in a three-dimensional (3-D) spherical shape with viscoelastic consistency and sufficient integrity for easy manipulation and handling during a bio-engineering process, such as tissue or organ engineering. Sufficient integrity means that the multicellular aggregate, 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.

[00020] A multicellular aggregate may be composed of any one or more pre-selected cell types. In general, the choice of cell type will vary depending on the desired three-dimensional biological tissue. For example, a multicellular aggregate used to engineer a comestible (edible) meat tissue may be formed of a cell type or cell types typically found in meat (e.g., muscle cells, etc.). Other cell types may be used to form the multicellular aggregate. One skilled in the art will be able to choose an appropriate cell type or types for the multicellular aggregate based on the intended use for the multicellular aggregate. Non-limiting examples of suitable cell types include contractile or muscle cells (e.g., striated muscle cells, including myoblasts and cardiomyocytes, and smooth muscle cells), neural cells, fibroblasts, connective tissue cells (including the cell types which make up bone and cartilage, cells capable of differentiating into bone forming cells and chondrocytes, and cell types which make up lymph tissues), parenchymal cells, epithelial cells (including endothelial cells that form linings in cavities and vessels or channels, exocrine and endocrine secretory epithelial cells, epithelial absorptive cells, keratinizing epithelial cells, and extracellular matrix secretion cells), hepatocytes, and undifferentiated cells (such as embryonic cells, stem cells, and other precursor cells), among others. For example, the cells used to form the multicellular aggregate can be obtained from a live human or animal subject and cultured as a primary cell line.

[00021] A multicellular aggregate may be homocellular or heterocellular. In homocellular multicellular bodies, the plurality of living cells includes a plurality of living cells of a single cell type. Almost all of the living cells in a homocellular multicellular body are cells of the single cell type, subject to some tolerance for low levels of impurities including a relatively small

number of cells of a different cell type that have no more than a negligible impact on the maturation of a construct including the homocellular multicellular body.

[00022] In contrast, a heterocellular multicellular body may include a significant number of cells of more than one cell type. For example, a multicellular aggregate can comprise a plurality of living cells of a first type and a plurality of living cells of a second type (etc.), the second cell type being different from the first cell type. Heterocellular multicellular aggregates can also include a plurality of cells of a first cell type, a plurality of cells of a second cell type, and a plurality of cells of a third cell type with each of the first, second and third cell types being different from the others of the first, second, and third cells types. The living cells in a heterocellular aggregate 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 self 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 center of a heterocellular multicellular aggregate generally have a stronger adhesion strength (and thus higher surface tension) than cells that sort to the outside of the multicellular aggregate.

[00023] Furthermore, when a heterocellular multicellular aggregate is composed of cells from tissues that are neighbors in normal development, in the course of sorting they may recover their physiological conformation. Thus, heterocellular multicellular aggregates may comprise a sort of pre-built internal structure, based on the adhesive and cohesive properties of the component cells, and the environment in which the cells are located. This can be used to build more complex biological structures. The composition of the multicellular aggregate (e.g., ratios of the various different cell types to one another) may be varied, including varying the cell number and size, to vary the size of the multicellular aggregate. As another example, heterocellular multicellular aggregates can include a plurality of living cells of a first cell type, a plurality of cells of a second type, and a plurality of cells of a third type. Again, self-sorting of the cells may occur in such heterocellular multicellular aggregates. Thus, when these multicellular aggregates are used to build a three-dimensional biological structure, for example a tubular structure, in the course of structure formation, these cell types may sort such that the endothelial cells line the internal structure of the tube (i.e., the lumen), the smooth muscle cells form a layer substantially surrounding the endothelial cells, and the fibroblasts form the outer layer of the tubular structure, substantially surrounding both the layer of endothelial cells and the layer of smooth muscle cells.

[00024] In general, the multicellular aggregates described herein 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 aggregates may contain various ECM proteins (e.g., gelatin, fibrinogen, fibrin, collagen, fibronectin, laminin, elastin, and/or proteoglycans). The ECM components or derivatives of ECM components are produced by the cells during the formation of the spherical multicellular aggregate, as discussed in further detail below. Thus, the ECM components or derivatives of ECM components are typically secreted by the cells in the multicellular aggregate. The methods described below typically allow secretion of a larger amount of ECM than possible with prior art methods of forming multicellular aggregates. For example, the amount of ECM in a spherical multicellular aggregate by volume may be >10% of the volume of the spherical aggregate, greater than about 20%, greater than about 30%, greater than about 40%, greater than about 50%, between about 10%-50%, between about 10%-40%, between about 10%-30%, between about 20%-50%, between about 20%-40%, between about 20%-30 %, etc. In contrast in prior art multicellular aggregates, the amount of ECM is virtually negligible (e.g., <10%).

[00025] In general, the amount of ECM in the multicellular aggregates described herein may be further enhanced by adding one or more inducers of ECM. In addition, the cells forming the multicellular bodies may be chosen specifically to enhance the amount of ECM. For example, the cells used to make the multicellular aggregate can be genetically manipulated by any suitable method known in the art to vary the expression level of one or more ECM components or derivatives of ECM components and/or one or more cell adhesion molecules or cell-substrate adhesion molecules (e.g., selectins, integrins, immunoglobulins, and cadherins). The ECM components or derivatives of ECM components may promote cohesion of the cells in the multicellular aggregate. In some variations a compound such as ascorbic acid, which is known to enhance ECM (e.g., fibrillar collagen) may be added.

[00026] The spherical multicellular aggregates described herein may be of various sizes within the scope of the invention. For example, a multicellular aggregate is typically a lumenless body, meaning that there is no open passage extending through the multicellular aggregate. The multicellular aggregate suitably has substantially no voids, hollow spaces or the like within the body.

[00027] The multicellular aggregate may be configured so none of the living cells therein is more than about 250 microns from an exterior surface of the multicellular aggregate, and more suitably so none of the living cells therein is more than about 200 microns from an exterior of the multicellular aggregate. Because of the proximity of the cells in the central portions of the multicellular aggregate to the exterior surface of the multicellular body, cells in the multicellular

aggregate can be supplied with oxygen and/or nutrients by diffusion thereof from a void space at the exterior surface of the multicellular aggregate toward the central portions of the body. Although there may be some necrosis of cells in one or more portions of the multicellular body (e.g., the central portion), the necrosis is limited, particularly as compared to prior art multicellular aggregates, in which the cells in the center of the aggregate may be more densely packed. The aggregates described here are typically loosely packed within the spherical aggregate, since, unlike prior art aggregates, the spherical aggregates are formed without centrifugation of the cells to form the spherical aggregate.

[00028] The cells used to form the multicellular aggregate may be cells of a specific type (e.g. smooth muscle cells, fibroblasts, etc.) that are cultured using standard tissue culture techniques.

Methods of preparing substantially spherical multicellular aggregates having endogenous ECM

[00029] In some variations, a method of preparing substantially spherical aggregates that produce their own ECM includes at least two steps: (1) allowing the cells to form a confluent layer (forming a confluent sheet) on a substrate; and (2) removing the sheet or layer from the substrate and allowing it to ball up into a substantially spherical aggregate.

[00030] Prior art methods of making multicellular aggregates limited the amount of time that the cells forming the aggregate could produce their own ECM. In contrast, the methods described herein permit the cells to being producing ECM from the start of culture (e.g., at t=0), and continue to produce ECM through the process.

[00031] For example, cells may be initially grown to confluence as a confluent layer. Smooth muscle cells (SMC) or fibroblasts grown on a substrate (such as a dish or plate) that allow the cells to adhere as they are grown, the cells will begin to produce ECM (mostly collagen). The cells may secrete the ECM molecules mostly on the top of the sheet, since cells will adhere to the bottom of the plate. Although the cells are adherent, they may begin to detach since the grown confluent sheet is under tension, and the cells may be driven to minimize the surface area.

[00032] While being cultured, the cells may produce ECM even without the addition of an inducing agent, however, inducing agents may be used. For example, ascorbic acid may be used to stimulate the cells to produce ECM at an elevated rate. For example, 50 μg/ml of ascorbic acid may be included in the culture medium trigger production of collagen.

[00033] In one example, cells (e.g., smooth muscle cells) are seeded onto an adherent substrate (e.g., culture dish) at a density high enough to obtain a confluent monolayer at the bottom of the wells of the multi-well plate culture dish (e.g., $1x10^5$ to $2x10^5$ cells/cm^2).

[00034] The cells may be of any appropriate source, including cultured cells. For example, smooth muscle cells may originate from cultures grown using standard tissue culture techniques,

and harvested from Petri dishes, tissue culture flasks or roller bottles by trypsin and used (possibly in combination with other cell types) to prepare cellular solutions or mixtures of appropriate composition. The cell concentration may be determined using a hemacytometer.

[00035] In this example, the conventional two-dimension monolayer cell culture is exposed to standard culture medium supplemented with collagen inducers (50 μg/ml of ascorbic acid alone; 50 μg/ml of ascorbic acid plus Ficoll 70 (37.5mg/ml) and Ficoll 400 (25mg/ml)). After approximately 10 days, the smooth muscle cells have formed multilayers, comprising cells and ECM, at least partially detach from the wells, around their periphery, as shown in FIG. 1.

[00036] In this example, the sheet formed could be manually peeled off from the culture flask and folded on itself, as illustrated in FIGS. 2A and 2B. As the cells grow in culture on the dish, the sheet or layer is begins to detach from the bottom of the culture dish. In FIG. 2A, the edges have begun to peel up, because of the tension on the layer. In FIG. 2B the layer has been completely removed from the bottom of the dish, and placed on a non-adherent substrate for further culture; the sheet has balled-up.

[00037] The sheet (layer) of cells is under tension due to the higher concentration of collagen on the top (the side of the sheet exposed to the medium). Ficoll may also be added to the medium, so the sheet is more homogeneous and collagen forms more evenly. As mentioned above, the folded sheet may be transferred in a new well on top of an agarose layer in supplemented medium, as shown in FIG. 2B.

[00038] In the absence of attachment to a substrate, the sheet reorganizes into the lowest energy configuration, and progressively rounds up into a substantially spherical shape. In one example, when the sheets were formed in a 24 well plate with bovine fibroblasts, they rounded up in 7 days and had a diameter of ~1mm, as illustrated in FIG. 3. The diameter may depend on the initial cell concentration in the well. These aggregates express large amounts of ECM appropriate to the type(s) of cells forming the aggregate, as illustrated below.

[00039] Once formed, these substantially spherical aggregates may be used for virtually any intended use in which multicellular aggregates would be beneficial, including engineered tissues.

[00040] In some variations of the methods described herein, one or more of these steps may be automated and/or the process may be scaled up significantly. For example, the methods described herein are amenable to scaling. In addition, stem cells or other sources of cells may be used to enhance industrial applicability of these methods and multicellular aggregates.

[00041] For example, the aggregate preparation may be at least partially automated by distributing cell suspension in the wells of multiwall culture dishes using multi-channel pipettes or fluid dispensers. For a more reproducible and accurate number of cells, a system similar to a

high speed FACS (up to 70,000 cells/s) could be used. Robotic protocols may be set up for medium change using, for example, multiplate washers.

[00042] In some variations, detaching of the cell sheets and transferring them to the non-adherent surfaces (e.g. agarose bed) may be automated. For example, the detachment of the sheets can be automated by using plates with Nunc UpCell™ surface, as illustrated in FIG. 4. (see also, http://www.nuncbrand.com/us/page.aspx?ID=11850#details). In this embodiment, lowering the temperature of the plates causes the sheets to be freed from the plate. The plates can be put upside down and the content of the wells transferred into new plates treated to avoid cell adhesion; the new plates may be coated with a layer of agarose.

Morphology of substantially spherical multicellular aggregates having endogenous ECM

[00043] Multicellular aggregates formed as described above were grown until they formed substantially spherical multicellular aggregates as described above (e.g., 10 days as a layer, followed by transfer of the layer to a non-adherent substrate and allowed to grow another 7 days), and examined. FIGS. 5A and 5B shows the results of a histological examination of aggregates that were fixed and prepared for histology. For example, FIG. 5B shows section that was stained for collagen (Picrosirius red; PCR staining) showed organized collagen inside the aggregates. FIG. 5A shows H&E staining. The aggregate in this example is composed of human aortic smooth muscle cells.

[00044] The aggregates formed as described herein are also competent to fuse, and thus viable for use as multicellular aggregates appropriate for forming engineered tissue or engineered tissue products. For example, layers or sheet formed of the substantially spherical multicellular aggregates may be used to form engineered tissue including engineered meats. FIG. 6 shows a time progression of two aggregates placed adjacent to each other and allowed to fuse. Any number of aggregates may be positioned adjacent to each other and fused in this manner.

[00045] For example, the substantially spherical multicellular aggregates described above may be used to form a thick layer by depositing a plurality of these aggregates onto a non-adhesive surface such as an agarose pad, or agarose-coated surface. The aggregates may then fuse into a sheet like structure. This sheet will have a substantial amount of ECM, since the ECM extruded during the formation of the individual aggregates will be incorporated into the sheet-like structure. Such sheets may be used, for example, to form engineered meats by stacking the sheets and allowing them to fuse, as described in US 13/558,928, filed 7/26/2012 (and titled "Engineered Comestible Meats"). The substantially spherical multicellular aggregates described herein may be particularly advantageous in forming sheet-like structures because the large amount of ECM may reduce the shrinkage (contraction) when forming sheets of material.

[00046] As used herein in the specification and claims, including as used in the examples and unless otherwise expressly specified, all numbers may be read as if prefaced by the word "about" or "approximately," even if the term does not expressly appear. The phrase "about" or "approximately" may be used when describing magnitude and/or position to indicate that the value and/or position described is within a reasonable expected range of values and/or positions. For example, a numeric value may have a value that is +/- 0.1% of the stated value (or range of values), +/- 1% of the stated value (or range of values), +/- 2% of the stated value (or range of values), +/- 5% of the stated value (or range of values), +/- 10% of the stated value (or range of values), etc. Any numerical range recited herein is intended to include all sub-ranges subsumed therein.

CLAIMS

What is claimed is:

1. A method of forming a multicellular aggregate, the method comprising:
   forming a confluent layer of cells on a substrate so that the layer is at least partially adherent to the substrate;
   removing the confluent layer of cells from the substrate so that it forms a balled up layer of cells; and
   culturing the balled up layer of cells on a non-adhesive substrate to form a substantially spherical multicellular aggregate.

2. The method of claim 1, wherein forming the confluent layer of cell comprises forming a confluent layer of homocellular cells.

3. The method of claim 1, wherein forming the confluent layer of cells comprises incubating the layer of cells with an agent that induces or enhances formation of extracellular matrix (ECM) from the cells.

4. The method of claim 3, wherein the agent is ascorbic acid.

5. The method of claim 1, wherein forming the confluent layer of cells comprises incubating the cells for more than 7 days.

6. The method of claim 1, wherein removing the confluent layer of cells comprises peeling the confluent layer of cells from the substrate.

7. The method of claim 1, wherein forming the confluent layer of cells comprise forming the confluent layer of cells on an adhesive substrate.

8. The method of claim 1, wherein removing the confluent layer of cells from the substrate so that it forms a balled up layer of cells comprises suspending the layer of cells.

9. The method of claim 1, wherein culturing the balled up layer of cells on a non-adhesive substrate to form a substantially spherical multicellular aggregate comprises culturing for greater than 5 days.

10. The method of claim 1, wherein culturing the balled up layer of cells on a non-adhesive substrate to form a substantially spherical multicellular aggregate comprises culturing

until greater than 10% by volume of the spherical multicellular aggregate is extracellular matrix (ECM).

11. A method of forming a substantially spherical multicellular aggregate having greater than 10% extracellular matrix (ECM) by volume, the method comprising:

forming a confluent layer of cells on a substrate;

removing the confluent layer of cells from the substrate;

transferring the layer of cells to a non-adhesive substrate to form a substantially spherical multicellular aggregate having greater than 10% ECM by volume.

12. A substantially spherical multicellular aggregate formed by the method of claim 1.

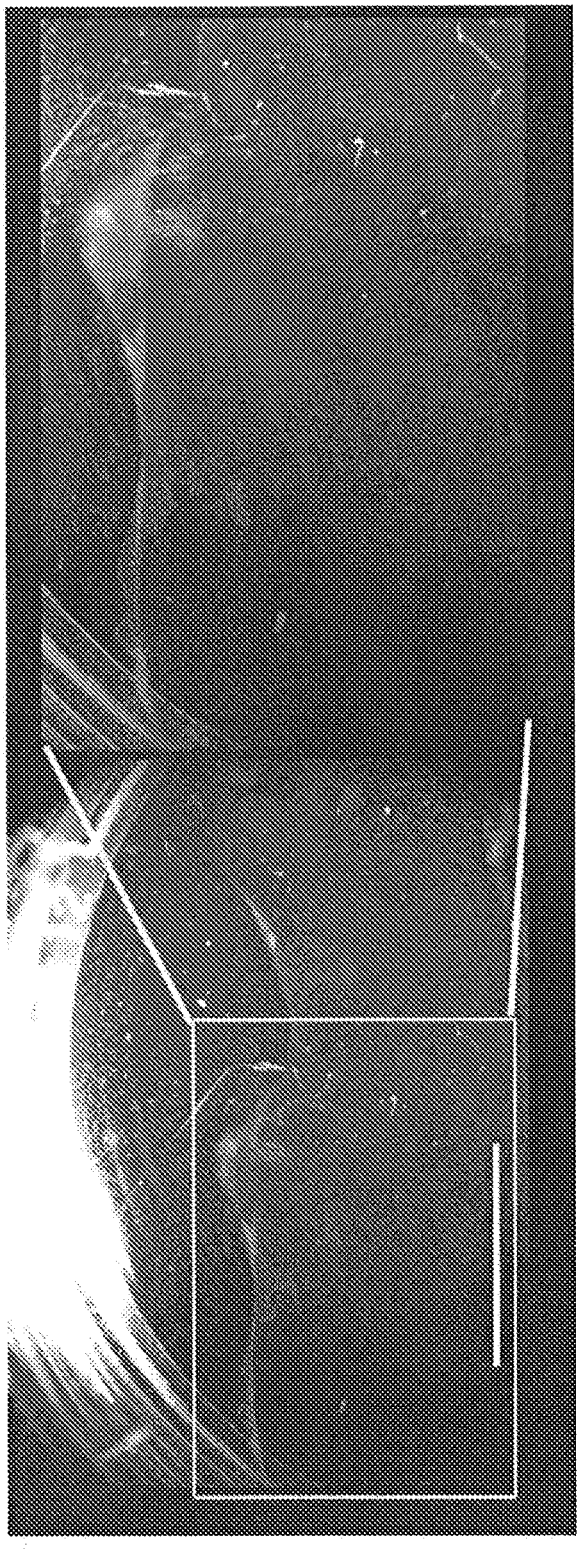

FIG. 1

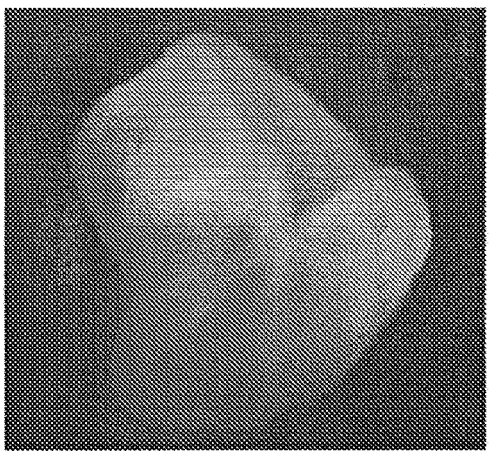

FIG. 2B

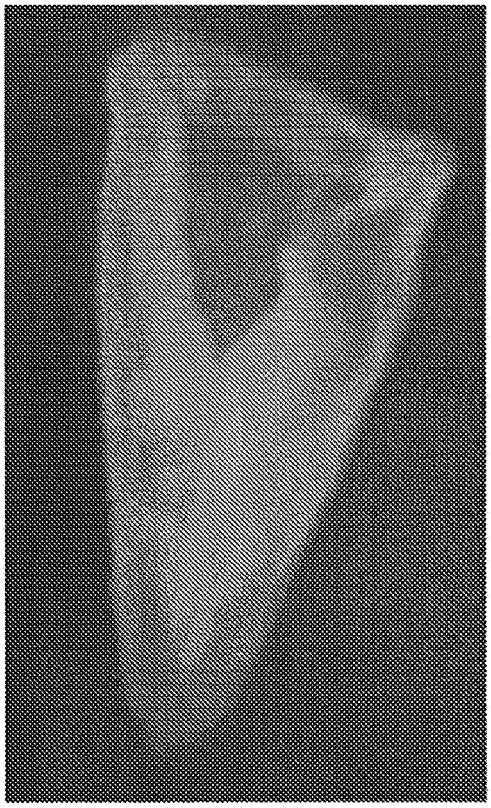

FIG. 2A

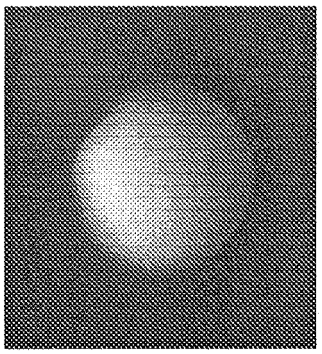

FIG. 3

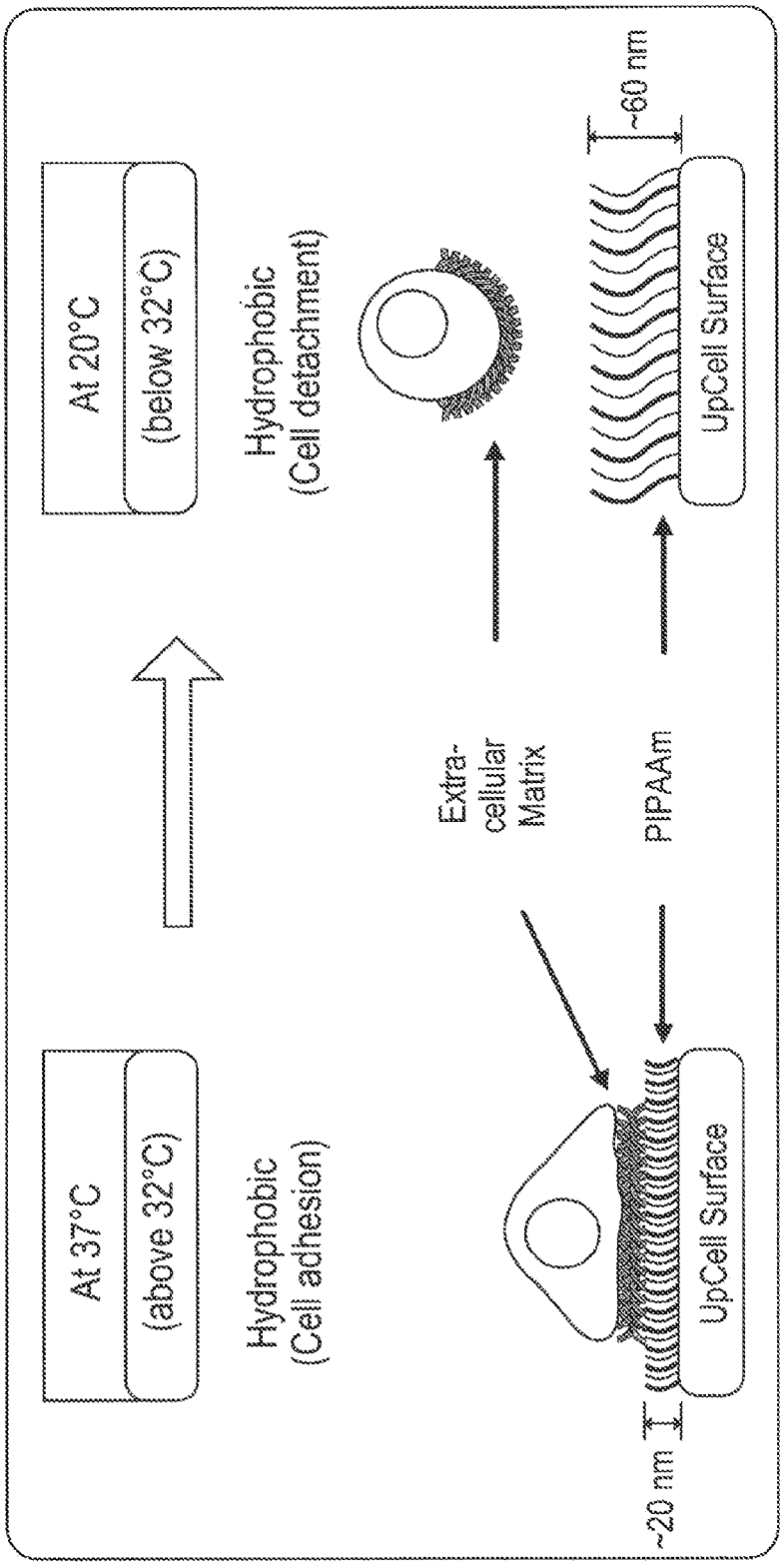
At 37°C
(above 32°C)
Hydrophobic
(Cell adhesion)
At 20°C
(below 32°C)
Hydrophobic
(Cell detachment)
Extra-
cellular
Matrix
PIPAAm
~20 nm
UpCell Surface
~60 nm
UpCell Surface

FIG. 4

FIG. 5B

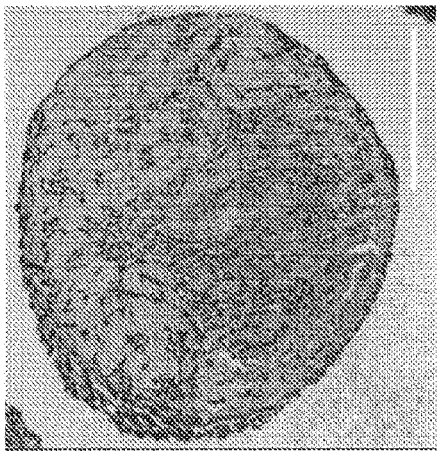

FIG. 5A

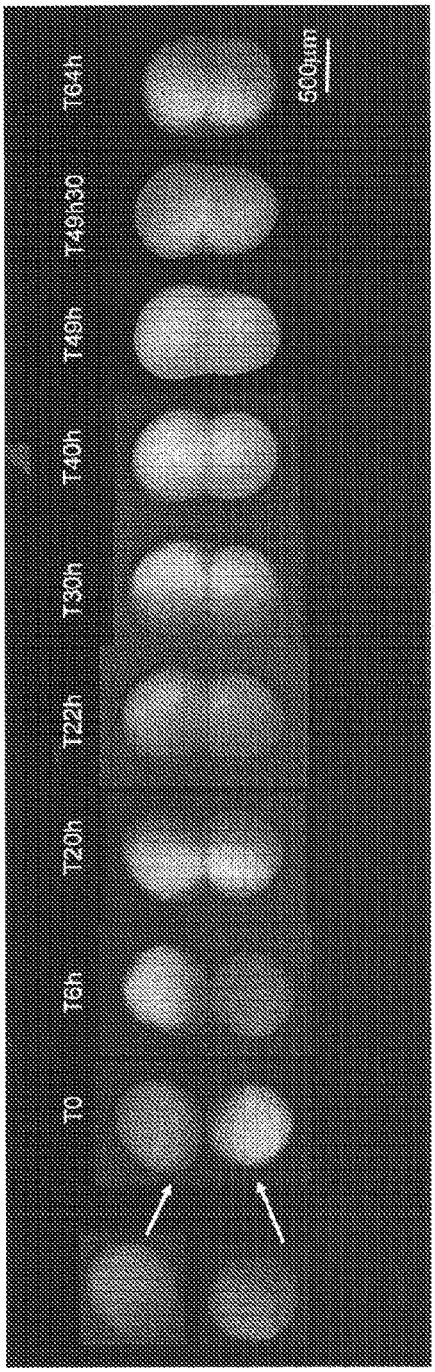

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/058684

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/00(2006.01)i, C12N 11/16(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 5/00; C12N 5/02; G01N 33/00; C12M 3/00; C12N 11/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: spherical multicellular aggregate, substrate, cells, confluent layer, extracellular matrix, culture

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2008-0070304 A1 (FORGACS, G. et al.) 20 March 2008 See abstract; paragraphs [0033] and [0047]; claims 1-2, 6 and 52-57.	1-12
Y	WO 2010-008905 A2 (THE CURATORS OF THE UNIVERSITY OF MISSOURI) 21 January 2010 See abstract; paragraphs [0059], [0062] and [00132]; claims 1-3, 21-23, 28-30 and 52.	1-12
Y	SOMMER, F. et al., `Ascorbic acid modulates proliferation and extracellular matrix accumulation of hyalocytes`, Tissue Engineering, June 2007, Vol. 13, No. 6, pp. 1281-1289. See abstract.	3-4
A	NOROTTE, C. et al., `Scaffold-free vascular tissue engineering using bioprinting` Biomaterials, October 2009, Vol. 30, No. 30, pp. 5910-5917. See abstract; page 4, paragraphs 4-7; figures 3-6.	1-12
A	MARGA, F. et al., `Developmental biology and tissue engineering`, Birth Defects Research (Part C) Embryo Today, December 2007, Vol. 81, No. 4, pp. 320-328. See abstract; figures 1 and 3.	1-12

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

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 2013 (20.12.2013)	**23 December 2013 (23.12.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. +82-42-472-7140

Authorized officer
HEO, Joo Hyung
Telephone No. +82-42-481-8150

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/058684

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2008-0070304 A1	20/03/2008	US 2012-288938 A1	15/11/2012
		US 8241905 B2	14/08/2012
		WO 2005-081970 A2	09/09/2005
		WO 2005-081970 A3	02/04/2009
WO 2010-008905 A2	21/01/2010	AU 2009-271223 A1	21/01/2010
		AU 2009-271223 B2	16/05/2013
		CA 2729559 A1	21/01/2010
		CN 102124331 A	13/07/2011
		EP 2310847 A2	20/04/2011
		EP 2310847 A4	13/02/2013
		JP 2011-525376 A	22/09/2011
		KR 10-2011-0033237 A	30/03/2011
		US 2010-0041134 A1	18/02/2010
		US 2012-196343 A1	02/08/2012
		US 8143055 B2	27/03/2012
		WO 2010-008905 A3	27/05/2010