FORMING CELL STRUCTURE WITH TRANSIENT LINKER IN CAGE

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. provisional application No. 60/960,743, filed October 11, 2007, the entire contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to formation of cell structures, particularly to methods and devices for forming three-dimensional (3D) cell structures.

BACKGROUND OF THE INVENTION

[0003] Three dimensional (3D) cell structures are important as they can mimic cell behaviors *in vivo*, more closely than a 2D cell culture. For example, 3D *in vitro* tissue models with high fidelity to *in vivo* tissues have important applications in tissue engineering and pathological model development, and can be used to study and test effects and mechanisms of potential therapeutic agents.

[0004] A difficulty in conventional techniques of forming 3D cell cultures is to provide efficient transportation of matter, such as a cell culturing medium, through the inner regions of 3D cell structures. As transportation of matter through a cell structure is typically by perfusion, it is more difficult to transport matter through the cell structure when the cell structure has a larger volume. In some conventional techniques, to form micro-scale cell structures, cells are encapsulated in an extra-cellular support, such as in a hydrogel or a thin layer of matrix which provides a 3D extra-cellular matrix (ECM) support for the cell structure. This extra-cellular support forms a barrier which limits transportation of matter to the cells.

SUMMARY OF THE INVENTION

[0005] It is thus desirable to form cell structures in an environment that facilitates transportation of matter through the cell structures. It has been discovered that a micro-scale cell structure can be efficiently formed with a transient linker and a cage in a fluid conduit. The transient linker initially links the cells to form cell aggregates but will not form a permanent barrier that limits transportation of matter to and from the cells. The cage retains the aggregated cells but has distributed openings to facilitate transportation of matter through different regions in the formed cell structure. The cage may include a plurality of micro-pillars arranged in a substantially U-shaped pattern, where the gaps between adjacent pillars allow fluid communication but are sized to retain aggregated cells.

[0006] According to an aspect of the present invention, there is provided a method of forming a cellular structure. The method comprises supplying cells and a transient linker to a volume partially enclosed by a cage. The linker facilitates initial attachment of adjacent cells to form a cell aggregate. The cage defines distributed openings that are sized to retain the cell aggregate. A fluid comprising a cell culture medium is supplied to the volume. The fluid is withdrawn from the volume through the openings. Aggregated cells retained in the volume are cultured to form a cell structure. The cells may be suspended in the fluid and the linker may be dissolved in the fluid before the fluid is supplied to the volume. A flow of the fluid through the volume may be maintained. The cells in the fluid may have a density of about 5 to about 6 million cells/ml and the transient linker in the fluid may have a concentration of about 6 to about 8 μM. The openings may be distributed to facilitate perfusion of the cell culture medium through the cell structure. The cage may be disposed in a conduit, and the fluid may flow through the conduit. The conduit may comprise a bottom and opposing side walls extending from the bottom. The cage may comprise a plurality of projections extending from the bottom and between the side walls. The projections may comprise micro-pillars. The micro-pillars may be arranged in a substantially U-shaped pattern. The gap between two adjacent micro-pillars may be about 10 to about 50 micrometers. The linker may comprise a polyethyleneimine backbone and hydrazide groups bonded to the backbone. The linker may have a molecular weight of about 2000 to about 20000 Dalton. The cells

may comprise HepG2 cells or rat bone marrow stem cells. The cells may comprise an aldehyde group. The cells may comprise cells that have been modified to form aldehyde groups on surfaces of the modified cells. The flow of the fluid may be actuated by applying a withdrawal force downstream of the distributed openings.

[0007] According to another aspect of the present invention, there is provided a cell culturing device. The device comprises a conduit; a fluid flowing in the conduit, the fluid comprising cells, a transient linker and a cell culture medium, the linker facilitating initial attachment of adjacent cells to form a cell aggregate; and a cage in the conduit for retaining aggregated cells formed in the fluid, the cage defining distributed openings that allow the fluid to flow through. The cage may comprise a plurality of projections, arranged in a substantially U-shaped pattern. The projections may comprise micro-pillars. The conduit may have a bottom and opposing side walls extending from the bottom, and the projections may extend from the bottom. The cells in the fluid may have a density of about 5 to about 6 million cells/ml and the transient linker in the fluid may have a concentration of about 6 to about 8 μM. The cells may be suspended in the fluid. The linker may be dissolved in the fluid. The linker may comprise a polyethyleneimine backbone and hydrazide groups bonded to the backbone. The linker may have a molecular weight of about 2000 to about 20000 Dalton. The cells may comprise an aldehyde group. The cells may comprise HepG2 cells or rat bone marrow stem cells. The fluid may be actuated by a withdrawal force applied downstream of the distributed openings.

[0008] Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] In the figures, which illustrate, by way of example only, embodiments of the present invention,

[0010] **FIG. 1** is a perspective view of a fluid conduit for forming a cell culture structure, exemplary of an embodiment of the present invention;

[0011] **FIGS. 2** and **3** are top plan views of the fluid conduit of **FIG. 1** during use;

[0012] **FIG. 4** is a confocal image of a representative 3D cellular aggregate formed according to an embodiment of the present invention;

[0013] **FIG. 5** is a scanning electron micrograph (SEM) of a representative 3D cellular aggregate formed according to an embodiment of the present invention;

[0014] **FIG. 6** is a transmission light image of a representative cell structure formed in a micro-fluid channel according to an embodiment of the present invention;

[0015] **FIG. 7** is a transmission light image of a comparative cell structure formed in the micro-fluid channel of **FIG. 6** but under different conditions;

[0016] **FIGS. 8** to **11** are confocal images of representative cell structures formed at different flow rates;

[0017] **FIG. 12** is a bar graph showing the dependence of the percentage of viable cells on the flow rate;

[0018] **FIG. 13** is a confocal image of a perfusion cell culture of HepG2 cells in a fluid channel, formed according an embodiment of the present invention;

[0019] **FIG. 14** is a transmission light image of the cell culture of **FIG. 13**;

[0020] **FIG. 15** is a confocal image of a perfusion cell culture of primary rat bone marrow stem cells in a fluid channel, formed according an embodiment of the present invention;

[0021] **FIG. 16** is a transmission light image of the cell culture of **FIG. 15**;

[0022] **FIG. 17** is a schematic diagram illustrating an alternative fluid conduit for forming a cell culture, exemplary of an embodiment of the present invention; and

[0023] **FIG. 18** is a perspective view of a further fluid conduit for forming a cell culture, exemplary of an embodiment of the present invention.

DETAILED DESCRIPTION

[0024] **FIGS. 1**, **2** and **3** show a fluid conduit **100** for forming and culturing a cell structure, exemplary of an embodiment of the present invention. Fluid conduit **100** may form or be part of a fluidic device for providing a fluid channel in the device. The device may have other components or features not shown in **FIGS. 1**, **2**, and **3** for providing functionalities that may be needed for using the device in a particular application.

[0025] Fluid conduit **100** has a bottom **102**, opposing side walls **104** extending from bottom **102**, an inlet **106**, an outlet **108**, and a cover (not shown). Inlet **106** is in fluid communication with a fluid source (not shown) for supplying a fluid to conduit **100**. Outlet **108** is in fluid communication with a fluid receiver (not shown) for withdrawing the fluid from conduit **100**. Inlet and outlet **106**, **108** may also be in fluid communication with another fluid supplier or receiver respectively, through different input and output conduits (not shown, but see **FIG. 17**). The cover covers the top of conduit **100** to provide an enclosure.

[0026] The shape and size of the fluid channel defined by conduit **100** may be selected depending on the particular application, including the shape and size of the cell culture structure to be formed. For forming micro-scale cell cultures, the width and height of the fluid channel in conduit **100** may be less than 1 mm. For example, in some embodiments, conduit **100** may have a generally rectangular cross section with a height varying from about 50 to about 500 micrometers.

[0027] Conduit **100** may be formed of any suitable material depending on the particular application. For example, bottom **102** and side walls **104** (and the top) may be formed of glass, plastic or a polymer material, or a combination thereof. Suitable polymers may include polycarbonate, polyacrylic, thick-photo resist epoxy resin (e.g. compounds in the SU-8 series from MicroChem Inc., MA, US), polyoxymethylene, polyamide, polybutylenterephthalate, polyphenylenether, polydimethylsiloxane (PDMS), mylar, polyurethane, polyvinylidene fluoride (PVDF),

PMMA (polymethyl methacrylate, flourosilicone, or combinations and mixtures thereof. The polymer may be formed using a suitable polymerisable material, which may include monomers, oligomeric building blocks, or any suitable precursor molecules. Different parts of conduit **100** may be formed of the same or different materials.

[0028] A plurality of micro-pillars **110** are disposed in conduit **100**, which are arranged in a substantially U-shaped pattern and spaced from side walls **104**. The open end of the U-shaped pattern faces inlet **106** and the partially-closed-end of the U-shaped pattern faces outlet **108**. The gaps between adjacent micro-pillars **110** may be in the range of about 10 to about 50 micrometers. The size of the gaps may be selected to provide a desired perfusion rate through the gaps, as will be further explained below. Micro-pillars **110** may have any suitable cross-sectional shape. The width of micro-pillars **110** may vary, such as in the range of 10 to 50 micrometers. The height of the pillars may be selected depending on the desired cell structure to be formed. In some embodiments, for example, the pillar height may vary from about 10 to about 500 micrometers. In some embodiments, the pillar may extend over the full height of conduit **100**.

[0029] The micro-pillars may be formed from the same material as that of conduit **100** or a different material.

[0030] Micro-pillars **100** define a partially enclosed volume or cell-growth region **112**, as delineated by the dash lines in **FIGS. 1** and **2**.

[0031] Optionally, pillars **100** may extend to the top cover of conduit **100**. Alternatively, cross-bars (not shown) may be provided to connect the top ends of opposing pairs of pillars **110**, and the cross-bars may be spaced from the top cover of conduit **100**.

[0032] In one embodiment, bottom **102** is made of glass, and the other parts of conduit **100** including side walls **104**, pillars **110**, and the top cover are formed from the same polymer. Side walls **104**, pillars **110**, and the top cover may be formed as an integral unit. In this case, pillars **110** extend from the top cover down to the glass bottom **102**. The polymeric portion of conduit **110** may be formed by

molding. The mold may be a microfabricated silicon mold. The polymeric portion may be tightly attached to the glass bottom **102** to form an enclosed fluid channel. The polymer and glass may be attached to each other by permanent chemical bonding or using a removable fastener such as clamps.

[0033] In another embodiment, conduit **100** may as a whole be formed of an integrated unit. The unit may be formed of a plastic, polymer, or the like.

[0034] Fluid conduit **100** and micro-pillars **110** may be fabricated using any suitable micro-fabrication technique. The surfaces of fluid conduit **100** and micro-pillars may be treated using known techniques to improve performance in a given application. For example, fluid conduit **100**, including micro-pillars **110**, may be fabricated using a micro-fabrication technique such as micromachining, replica moulding, soft lithography, reactive ion etching (RIE), or deep reactive ion etching (DRIE), or the like.

[0035] In one embodiment, conduit **100** may be formed using polydimethylsiloxane (PDMS). A silicon template may be first formed by deep reactive ion etching. The PDMS material is then molded in the silicon template. The molded PDMS structure may then be oxidized in oxygen plasma, such as for about 1 minute, to chemically bond the PDMS structure to a cover plate (not shown), such as a glass cover slip. The device may be sterilized before use.

[0036] In use, as better illustrated in **FIGS. 2** and **3**, a fluid **114** is caused to flow through conduit **100** and cell growth region **112**.

[0037] Fluid **114** contains a cell culture medium and a transient linker for initially linking the cells to form cell aggregates. The transient linker will only temporarily attach to the cells and will dissociate from the cells after the adjacent cells form cell aggregates. The linker may be a transient inter-cellular polymeric (TIP) linker. The linker may be dissolved in fluid **144**. Cells **116** for forming a desired cell structure are suspended in fluid **114**. Fluid **114** may include a carrying solvent for dissolving and carrying the TIP and other ingredients therein. Fluid **114** may be prepared using any suitable preparation technique known to persons skilled in art.

[0038] The cell culture medium may contain any suitable material(s) for culturing the particular cells used, as can be appreciated by persons skilled in the art. For example, culture medium may include the nutrients required or desirable for cell growth and culture. For example, the cell culture medium may contain Dulbecco's modified Eagle medium (DMEM), Minimum Essential Medium (MEM), F-10 Nutrient Mixture, F-12 Nutrient Mixture, medium developed by Roswell Park Memorial Institute (RPMI medium), Iscove's Modified Dulbecco's Medium (IMDM), glucose, fetal calf serum (FCS), penicillin/streptomycin, CO_2, growth factors, or other substances.

[0039] Cells **116** may be cells that can form cell aggregates through a transient linker, and may include one or more types of cells. For example, cells with sialic acid residues, which can be modified by $NaIO_4$, may be suitable. In some embodiments cells with a higher expression of sialic acid may be used. For example, cell lines such as HepG2 human liver cell line, A549 human lung epithelial cell line, HeLa human cervical cell line, human glioma cell lines U87 and U251, or the like may be suitable. Primary cells such as rat bone marrow mesenchymal stem cells and porcine hepatocytes may also be suitable.

[0040] The cells may be selected so that they can be seeded in cell growth region **112** as fluid **114** flows through conduit **100**. Cells **116** may be surface-modified so that they will aggregate through the transient linker. For example, cells **116** may be cells that contain, or have been modified to contain, aldehyde groups. Cells **116** may be modified from HepG2 cells (human liver cell line), primary rat bone marrow stem cells (BMSC), A549 human lung epithelial cell line, HeLa human cervical cell line, human glioma cell lines U87 and U251, primary porcine (pig) hepatocytes, or other types of cells.

[0041] For culturing HepG2 and BMSC cells, the cell culture medium may include DMEM, 10% FCS and glucose. The glucose content in the cell culture medium may be higher for HepG2 cells, e.g. about 4.5 g/L; and lower for BMSC cells, e.g. about 1.0 g/L.

[0042] In one embodiment, it may be desirable to prevent substantial attachment of the cells to the wall surfaces, so that a desired flow rate may be

maintained in conduit **100**. Attachment may be avoided by either selecting cells that do not attach to the particular wall surfaces or adjusting the fluid flow rate in conduit **100** so that substantial attachment does not occur.

[0043] The culturing medium may be osteogenic and may be prepared from a basal medium with 100 nM dexamethasone, 0.05 mM ascorbic acid 2-phosphate and 10 mM β-glycerophosphate.

[0044] The transient linker is selected so that it can initially link cells **116** to attach them to each other and form cell aggregates **122**. The linker may be selected so that it can establish immediate contact between cells and cause the cells to aggregate to form 3D cellular aggregates. The linker molecules are only temporarily bonded with cells **116** and can dissociate from attached cells after the cells have attached to each other. The half-life of the linker attached to the cell surface may be in the range of about 1 to about 5 days. In one embodiment, the half-life may be about 12 hours or shorter.

[0045] For example, for cells with surface aldehyde groups, the linker may contain hydrazide terminal groups, which can react with the aldehyde groups to cause aggregation of the cells. In one embodiment, hydrazide groups may be conjugated or bonded to a polymer backbone such as a polyethyleneimine (PEI) backbone. In different embodiments, other linear polymeric linkers, dendrimer linkers, two-step linkers, or the like, may be used. Some suitable transient linkers are disclosed in, for example, Zhao *et al.*, "Dendrimer hydraides as multivalent transient inter-cellular linkers," *Biomaterials*, 29 (2008) 3693-3702; and De Bank *et al.*, "Surface engineering of living myoblasts via selective periodate oxidation," Biotechnology and bioengineering, vol. 81, 2003, pp. 800-808.

[0046] A transient linker will dissociate from the cells after the cells have formed aggregates. Conveniently, the transient linker will not form a permanent barrier around the cells. This allows efficient mass transportation to and from the cells. Further, this allows the cells to establish natural cell-cell interaction, to secrete ECM, and to establish cell-matrix interaction, which may be desired for a 3D inter-cellular support.

[0047] The linker may be based on non-toxic low molecular-weight PEI. In one embodiment, the linker may have a molecular weight from about 2000 to about 20000 Dalton. The primary amine groups on the PEI arms may be modified to yield hydrazides, which can react with the aldehyde handles on chemically modified cell surfaces to aggregate cells. The linker may be able to reside transiently on cell surfaces with a half-life of about two days. The linker may be selected to allow anchorage-dependent cells to produce their own native environment for 3D support without incorporating exogenous biomaterials that potentially hinders mass transport. As such, the cells can secrete and accumulate their own ECM for support.

[0048] Some cells need to be supported by, or anchored to, a substrate to survive, grow and multiply, which are referred to as anchorage-dependent cells. For example, mammalian cells (primary cells and cell lines) are anchorage dependent. Anchorage-dependent cells may be conveniently used in an embodiment of the present invention, as once the cells have aggregated and been confined in the cell-growth region, they are supported by neighboring cells. Thus, it is not necessary to anchor the cells to an external matrix support such as gels.

[0049] The temperature of the fluid may be maintained at a level suitable for culturing the particular cells. In one embodiment, the temperature may be at about 37 °C. The temperature in conduit **100** may be controlled using a heating device (not shown) and a temperature controller (not shown). In some embodiments, a heater (not shown) may be embedded in the fluidic device on which conduit **100** is provided.

[0050] The flow rates of the fluid flow through conduit **100** are controlled to allow cells **116** to form cell aggregates **118** initially linked by the TIP linker. The flux (the rate of fluid flow per unit area) should be selected to reduce or minimize the impact forces created by the fluid flow on the cells while maintaining sufficient supply of cell culture medium to aggregated cells trapped by micro-pillars **110**, in order to maintain viability of the cells. In some embodiments, it may reduce the flow impact if the fluid flow is actuated by applying a withdrawal force downstream of the distributed openings defined by micro-pillars **110**. For example, an active

withdrawing force may be applied through outlet **108**, such as using a fluid pump, instead of applying an actuating force at inlet **106**.

[0051] It should be understood that the local flux of fluid flow in conduit **100** may not be uniform and may vary in different regions in the channel and at different times. For example, as cell aggregates accumulate in cell growth region **112**, the fluid flow inside cell growth region **112** or through the gaps between micro pillars **110** may slow down with time. If a constant overall flow rate through conduit **100** is maintained, the fluid flow outside cell growth region **112** may become faster with time. However, in some embodiments and applications, it is still possible to control the mass transportation or diffusion rate in cell growth region **112** or in the gaps between pillars **110** by adjusting the overall flow rate or flux through conduit **100**.

[0052] As can be understood, a higher flux may provide faster diffusion in the growth region, but it may also increase the shear stress on the cells. Therefore, the overall flux may be adjusted and optimized to achieve a desired balance.

[0053] The flow rate or flux, and other operational parameters, may be optimized so that the *in situ* formed cellular aggregates are large enough to be confined by pillars **110**, but small enough to prevent clogging of the fluidic channel. The sizes of the cellular aggregates may be modulated by cell density and the inter-cellular linker concentration in the fluid. In some embodiments, the cell density may be about 5 to about 6 million cells/ml and the inter-cellular linker concentration may be about 6 to about 8 μM. In one embodiment, the cell density may be about 6 million cells/ml and the inter-cellular linker concentration may be about 6 μM. If the cell density and linker concentration are too high, substantial clogging may occur at inlet **106**. If the cell density and linker concentration are too low, aggregation of cells may be too slow to efficiently trap cells in cell growth region **112**.

[0054] The gaps between micro-pillars **110** have sizes that are selected to allow fluid **114** and individual cells **116** to flow through, but to retain cell aggregates **118** within cell-growth region **112**. Gaps in the range of about 10 to about 50 micrometers have been found to be suitable for some cells.

[0055] As fluid **114** flows through conduit **110** and through the gaps between micro-pillars **110**, cell aggregates **118** continue to grow and eventually form a cell structure **120**, with a shape and dimensions generally defined by the locations and shapes of micro-pillars **110** and conforming to cell-growth region **112**. As can be appreciated, micro-pillars **110** can be used to confine the cellular aggregates to form cellular constructs of consistent dimensions.

[0056] Cell structure **120** may be cultured for a desired period of time, such as up to a few weeks, depending on the particular application. The contents of input fluid **114** and cell culture medium may be adjusted or varied during different stages of the formation and culturing process.

[0057] The cell growth in conduit **100** may be observed or monitored. For example, images of the cell aggregates or cell structure may be taken during culturing. For this purpose, at least one side of conduit **100** may be transparent. Images of the cells may be obtained using any suitable technique, such as confocal imaging, transmission light imaging, SEM, or the like.

[0058] For imaging, labeling or other purposes, the cells may be stained, such as by F-actin staining, immunostaining of E-cadherin, von Kossa staining, or the like. Staining may be performed *in situ*, by changing the contents of input fluid **114**, including adding appropriate staining materials to input fluid **114**.

[0059] The cell culture medium used in the cell culturing process may be optionally re-circulated. A closed-loop circulation system (not shown) may be provided and a multi-channel peristaltic pump may be used to circulate the fluid carrying the cell culture medium.

[0060] Images and test results of representative cell aggregates and cellular structures formed according to the process described above are shown in **FIGS. 4 to 16**, as further described below.

[0061] As can be appreciated, the openings or gaps between micro-pillars **110** can be conveniently utilized to perfuse the culture medium through various regions in cellular structure **120** to enhance mass transport to and in the structure.

[0062] Effectively, micro-pillars **110** form a cage in fluid conduit **100** for retaining aggregated cells **118** formed in fluid **114**.

[0063] In other embodiments, the cage for retaining aggregated cells may be formed differently. For example, the cage may be formed of pillars, bars, wires, or the like, or a combination thereof. The cage may have one or more open sides. In any case, a volume is partially enclosed by the cage. This volume will substantially define the shape of the cell structure formed from the retained cells. The cage should define distributed openings to allow fluid to flow through, and to facilitate perfusion of matters through the volume. However, at least the openings from which the fluid is withdrawn from the cage should be sized to retain aggregated cells. The distributed openings may be provided on two or more sides of the cage. The cage may be shaped so that the enclosed volume has a desired shape. In some embodiments, the cage may have an open side, as illustrated in **FIG. 1**. In other embodiments, the cage may be at least partially closed on all sides. One or more sides of the cage may be completely closed (see e.g. bottom **102** of conduit **100** in **FIG. 1**). As can be appreciated, the cells, transient linker and cell culturing medium may be supplied to the enclosed volume through distributed openings of a partially-closed side or through an open side of the cage. In some embodiments, the input fluid may be supplied to the volume through an opening at the bottom or at the top of the cage.

[0064] In some embodiments, the cage may be formed of a rigid material. In other embodiments, a portion of the cage may be formed of a flexible material. For example, a side of the cage may be formed of a flexible material, such as a net or a membrane. In some applications, the net or membrane may have a pre-defined profile when pressured by the fluid flow and the aggregated cells retained in the cage.

[0065] The cage may have a fully or partially closed top.

[0066] The cage may be formed or placed in a conduit. The conduit may have the shape of a channel or a chamber, or the like. The sides of the cage with distributed openings may be spaced away from walls of the conduit to allow

efficient fluid communication through the openings. The cage may be immersed fully or partially in the fluid carrying the cell culturing medium.

[0067] As now can be appreciated, the arrangement illustrated in **FIGS. 1** to **3** may be modified and still achieve some of the benefits or advantages mentioned herein.

[0068] For example, **FIG. 17** shows a fluid conduit **200**, with bottom **202**, side walls **204**, inlet **106**, outlet **208**, and pillars **210**. As for fluid conduit **100** described earlier, a fluid **214** flows through conduit **200**. Cells **218** thus form cell aggregates **218** and eventually a cell structure **220**. Inlet **206** is in communication with three input conduits, a central conduit **222** and two side conduits **224**. Similarly, outlet **208** is in communication with three output conduits, a central conduit **226** and two side conduits **228**.

[0069] In one embodiment, conduit **200** may have a length of about 10 mm, a width of about 0.6 mm, and a height of about 0.1 mm. Each micro-pillar **210** may have an elliptical cross-section as shown, with the long axis being about 0.5 mm and the short axis being about 0.03 mm. The gaps between adjacent pillars may be about 0.02 mm wide. The distance between the closed end and the open end of the U-shaped pattern may be about 0.2 mm. For this arrangement, the cell density in the seeding fluid may be about 1.5 to about 10 million cells/ml, depending on the cell size. With larger cells, the optimal cell density may be lower.

[0070] Elliptical pillars may be advantageous in some applications. However, the pillars may have other cross-sectional shapes in different applications or embodiments.

[0071] In use, central conduit **222** may be connected to a cell reservoir (not shown) for supplying cells **216** to conduit **200**. Side conduits **224** may be connected to a source of the culture medium for supplying the culture medium to conduit **200**. The transient linker may be dissolved in the culture medium. A 4-way valve (not shown) may be provided at inlet **206** for controlling fluid flow and feeding different materials to conduit **200**. For example, the valve connected to central

conduit **222** may be initially open and then closed after sufficient cells have been supplied to conduit **200**.

[0072] When cells are being supplied to conduit **200**, fluid **214** may be withdrawn from conduit **200** through side conduits **228**, such as using a syringe pump (not shown). During culturing of cell structure **220**, fluid **214** may be withdrawn from all of conduits **226** and **228**.

[0073] In a further variation of the embodiment shown in **FIG. 1**, micro-pillars **110** may be replaced by a cage structure that defines a partially enclosed volume and distributed openings. For example, instead of pillars, a cage wall with spaced slots or openings therein may be provided, as illustrated in **FIG. 18**.

[0074] The fluid conduit **300** shown in **FIG. 18** is similar to fluid conduit **100** of **FIG. 1**, having a bottom **302**, side walls **304**, inlet **306** and outlet **308**. The cage wall **310** is provided to replace pillars **110** for retaining aggregated cells. Distributed openings **312** are provided in wall **310** to allow the culture medium to flow through.

[0075] In another embodiment, the cage structure may include a fence-like screen or another type of filtering device for retaining aggregated cells while allowing fluid to be withdrawn through the screen or filtering device.

[0076] The micro-pillars may also be replaced with other projections extending from the bottom wall of the conduit. For example, micro-pillar arrays and the techniques for fabricating micro-fluidic devices disclosed in WO 2006/052223 to Yu *et al.*, entitled "Cell Culture Device" and published 18 May 2006, may be modified and adapted for use in forming a suitable device used in an embodiment of the present invention. The contents of WO 2006/052223 related to fabrication of a fluidic device are incorporated herein by reference.

[0077] The conduit or channel in which the cage is placed may have different shapes and sizes in different embodiments. For example, it is not necessary that side walls **104** are parallel.

[0078] In some embodiments, the conduit may have the shape of a chamber. The chamber may have a generally rectangular, cylindrical or spherical shape. The conduit may also be shaped or sized to accommodate other fluidic elements or devices in addition to the retention cage. The inlet and outlet of the conduit may be provided at any desired locations in the chamber. Fluid ports may also be provided in a wall, such as the bottom, side or top wall, of the conduit to provide alternative or additional inlet/outlet for fluid communication.

[0079] To promote formation of cell aggregates using the transient linker, the cells may have, or modified to have, reactive handles on cell surfaces. The transient linker may have corresponding terminal groups for reacting with the reactive handles to "glue" the cells together. The cell surfaces may be modified genetically, via enzymatic treatment, or chemically to generate the reactive handles. Exemplary techniques for modifying cell surfaces are disclosed for example in B. Kellam *et al.*, "Chemical modification of mammalian cell surfaces," *Chem. Soc. Rev.*, 2003, vol. 32, pp. 327-337; E. Saxon *et al.*; "Chemical and biological strategies for engineering cell surface glycosylation," *Annu. Rev. Cell. Dev. Biol.*, 2001, vol. 17, pp. 1-23; S-M. Ong *et al.*, "Transient inter-cellular polymeric linker," *Biomaterials*, 2007, vol. 28, pp. 3656-3667 (hereinafter referred to as "Ong"), the entire contents of each of which are incorporated herein by reference.

[0080] A suitable technique for forming a TIP linker is disclosed in Ong. Other TIP linkers or techniques for forming the TIP linker may also be used.

[0081] The embodiments and modification discussed herein are for illustration purposes and are not exhaustive. Other modifications are also possible.

[0082] The exemplary embodiments described herein can be advantageously used in many applications.

[0083] For example, with the combined use of a transient linker and a micro-pillar array, a 3D cellular structure may be formed quickly, such as within about 5 minutes, and with a precise shape and dimension. The formation process can be relatively easy to perform.

[0084] As no permanent extra-cellular matrix or bulk material is used to support the cells, sufficient and efficient transportation of matter through different regions in the cell structure is possible. Continued perfusion through the cell structure both during and after formation is possible due to fluid communication through the gaps between the pillars. The absence of a permanent external support such as a hydrogel matrix that encapsulates the cells and the continued fluid flow allow transport of matters such as oxygen and nutrients to the cells and transport of matters such as metabolic wastes from the cells.

[0085] The improved mass transport can be useful for delivery of biological agents to a cellular construct during certain applications, such as in drug testing or biological studies.

[0086] A high level of biomimicry may be achieved by allowing the cells to secrete and accumulate their own ECM to produce a native microenvironment without incorporating exogenous biomaterials and utilizing perfusion culture that mimics *in vivo* vascularization. A higher biomimicry can potentially assist the acquisition of more predictive results from *in vivo* cellular responses, such as in drug testing or biological studies.

[0087] A micro-scale 3D cellular structure formed according an embodiment of the present invention can exhibit many advantages over macro-scale cell structures. For example, the micro-scale 3D cellular structure is more compact in size, may have a higher disposability, can provide faster and parallel analyses, and can reduce the reagent volume required in many applications.

[0088] Embodiments of the present application may be used with a biomimicry microchip for drug testing applications. Various tissues may be produced by aggregating one or more cell types in a micro-channel. The use of a transient inter-cellular linker in place of permanent hydrogels for cellular support allows the cells to secrete and accumulate native ECM for support rather than relying on exogenous biomaterials. This cell construct therefore better mimics in vivo cell behaviors.

[0089] Multiple fluid channels may be provided to provide high-throughput, such as in drug testing. The micro-channels can be connected to gradient generators to enable simultaneous testing of a range of concentrations of drugs on the cellular structures in different micro-channels. The gradient generator can be designed to be linear, sigmoidal, or exponential, thus providing versatility depending on the user's needs. Such multiplexibility may be useful for modifying existing integrated fluidic circuits to provide efficient manipulation of fluids for high throughput drug testing applications.

[0090] Multiple fluid chips may be connected sequentially to perform a method described herein. Different chips may be used to culture different cell types, representing different tissue analogues. Alternatively, different cell types can be cultured in different chambers connected by micro-fluidic channels on a single chip. Therapeutic agents can be circulated through the tissue analogues and their effects at a systemic level can be assayed. Such an arrangement is potentially beneficial for evaluating candidates of therapeutic agents based on a systemic response, which is an order higher than a tissue response. This has potential usage in many different micro-fluidic devices.

[0091] With a transparent conduit material (such as a glass-bottom), the micro-meter scale 3D cell culture formed in the micro-channel can be readily imaged with existing imaging modalities at high resolution. For example, the cell culture may be imaged using phase contrast microscopy, confocal laser scanning microscopy, or two-photon laser microscopy. This enables real-time imaging of cells cultured in a physiological 3D microenvironment, e.g., for the study of various dynamic cellular processes such as epithelial cells polarization, protein trafficking, endocytosis, transcytosis, proliferation, apoptosis, or the like. In particular, it allows high-content screening of single cells in a 3D microenvironment for various applications, including drug testing. Embodiments of the present invention can allow imaging of mammalian cells cultured in 3D while controlling the micro-environmental fluid flow around the cells.

[0092] Embodiments of the present invention may also be used to model various *in vitro* disease developments, such as cancer development, liver and lung fibrosis, or viral infection. By introducing different cell types, or disease-causing

agents, a series of models representing different stages of a disease prognosis can be developed. Such models are useful for studying the underlying mechanisms of disease development and testing potential therapeutic agents for disease treatment.

[0093] EXAMPLES:

[0094] Example I

[0095] The cell surfaces of HepG2 cells (human liver cell line) were modified using $NaIO_4$ to generate aldehyde handles on the cell surfaces, by adding $NaIO_4$ to a cell suspension in a tube and incubating the resulting mixture for 15 minutes.

[0096] The modified cells were suspended in a culture medium. The cell density was about 5 to about 6 million cells/ml. The culture medium included DMEM, glucose, FCS, and penicillin/streptomycin.

[0097] A TIP linker was also dissolved in the culture medium. The TIP linker was a polymeric molecule formed of multiple hydrazides conjugated to a polyethyleneimine (PEI) backbone. The linker concentration was about 6 to about 8 μM.

[0098] A solution of the culture medium with the cells and TIP linker was passed through a micro-channel illustrated in **FIG. 17** at room temperature. The microfluidic channel had dimensions of 1 cm (length) × 0.6 mm (width) × 0.1 mm (height). The microfluidic channel had two inlets and one outlet. An array of 0.03 mm × 0.05 mm elliptical micro-pillars with 0.02 mm gaps was situated in the center of the microfluidic channel, defining a cell residence compartment (growth region) that was 0.2 mm wide.

[0099] The solution was passed through the channel at various flow rates The fluid flow was driven by withdrawal of the fluid at the outlet end of the micro-channel.

[00100] It was observed that aggregation of cells formed in the cell-growth region. A confocal image of a representative cell aggregate is shown in **FIG. 4**. To visualize the presence of the linker on the cell surfaces, a linker conjugated with a

fluorescent dye was used. The linker is shown in **FIG. 4** as white circular regions around the cells. As can be seen in **FIG. 4**, many cells were encapsulated by the linker molecules and some of these encapsulated cells were in contact with one another, illustrating three-dimensional inter-cellular support effected by the linker.

[00101] An SEM image of the cellular aggregate is shown in **FIG. 5**, with the transient linker being present but invisible to the eye.

[00102] After about five minutes, a cellular structure was formed in the cell-growth region defined by the micro-pillars. A transmission light image of a representative cell structure formed in the micro-channel at a flow rate of about 0.03 ml/h is shown in **FIG. 6**.

[00103] After seeding had been completed, a culture medium without the TIP linker and cells was flowed through the fluid channel. A bar-shaped cell structure with a generally rectangular cross-section was formed.

[00104] Example II

[00105] A comparative cell structure was formed as in Example I except that the aggregation conditions were different. In particular, the flow rate was relatively low for the given cell density and TIP linker concentration. **FIG. 7** shows a transmission light image of this cell structure. As can be seen, under these conditions, the gaps between the pillars were clogged by cell aggregates. As a result, the formed structure did not have well-defined shape and dimension.

[00106] Example III

[00107] Cellular structures were formed as in Example I, except that the flow rate was varied to determine the optimal perfusion flow rate for the particular device and cell samples tested.

[00108] Each cellular construct was formed by flowing the cell culture medium at a flow rate selected from 0.01, 0.03, 0.06, or 0.22 ml/hr for one day.

[00109] The resulting structures were assessed with fluorescence viability staining. The live cells were stained with Calcein AM. The dead cells were stained with propidium iodide.

[00110] **FIGS. 8**, **9**, **10** and **11** show confocal images of representative cell structures formed at the flow rate of 0.01, 0.03, 0.06 and 0.22 ml/hr respectively.

[00111] The viability of the cells at different flow rates is shown in **FIG. 12**. As can be seen, for this particular arrangement and these cells, the viability was highest (about 80%) at the flow rate of 0.03 ml/hr. At the other flow rates, the viability was about 50%.

[00112] Without being limited to any particular theory, it may be expected that at too high a flow rate, a high shear or pressure exerted on the cells can cause reduction in viability; and at too low a flow rate, insufficient mass transport (of nutrients) can cause reduction in viability.

[00113] Example IV

[00114] Anchorage-dependent cells (HepG2 and rat bone marrow stem cells) were used to form cellular structures in a similar procedure as described in Example I, with a flow rate of 0.03 ml/hr, and were cultured for three or 14 days. It was observed that the 3D cell structures were adequately supported in the micro-channel for a period of up to two weeks.

[00115] The viability of the cells were assessed using fluorescence viability staining. Maintenance of the 3D morphology of the structures was assessed using F-actin staining.

[00116] The results showed excellent viability and maintenance of 3D morphology after 3 or 14 days of culture, respectively.

[00117] **FIG. 13** is a confocal image of a representative cell structure formed of HepG2 cells after 3 days of culture. **Fig. 14** is an SEM image of the same cell structure in the micro-channel.

[00118] **FIG. 15** is a confocal image of a representative cell structure formed of primary rat BMSC cells after 14 days of culture. **Fig. 16** is an SEM image of the same cell structure in the micro-channel.

[00119] Confocal images of actin staining of the sample cell structures (not shown) also showed that the cortical actin distribution in the sample structures was typical of a 3-D morphology.

[00120] Other features, benefits and advantages of the embodiments described herein not expressly mentioned above can be understood from this description and the drawings by those skilled in the art.

[00121] Of course, the above described embodiments are intended to be illustrative only and in no way limiting. The described embodiments are susceptible to many modifications of form, arrangement of parts, details and order of operation. The invention, rather, is intended to encompass all such modification within its scope, as defined by the claims.

WHAT IS CLAIMED IS:

1. A method of forming a cellular structure, comprising:

   supplying cells and a transient linker to a volume partially enclosed by a cage, said linker facilitating initial attachment of adjacent cells to form a cell aggregate, said cage defining distributed openings that are sized to retain said cell aggregate;

   supplying a fluid comprising a cell culture medium to said volume;

   withdrawing said fluid from said volume through said openings; and

   culturing aggregated cells retained in said volume to form a cell structure.

2. The method of claim 1, wherein said cells are suspended in said fluid and said linker is dissolved in said fluid before said fluid is supplied to said volume.

3. The method of claim 1 or claim 2, comprising maintaining a flow of said fluid through said volume.

4. The method of claim 3, wherein said cells in said fluid have a density of about 5 to about 6 million cells/ml and said transient linker in said fluid has a concentration of about 6 to about 8 μM.

5. The method of any one of claims 1 to 4, wherein said openings are distributed to facilitate perfusion of said cell culture medium through said cell structure.

6. The method of any one of claims 1 to 5, wherein said cage is disposed in a conduit, and said fluid flows through said conduit.

7. The method of claim 6, wherein said conduit comprises a bottom and opposing side walls extending from said bottom.

8. The method of claim 7, wherein said cage comprises a plurality of projections extending from said bottom and between said side walls.

9. The method of claim 8, wherein said projections comprise micro-pillars.

10. The method of claim 9, wherein said micro-pillars are arranged in a substantially U-shaped pattern.

11. The method of claim 10, wherein a gap between two adjacent ones of said micro-pillars is about 10 to about 50 micrometers.

12. The method of any one of claims 1 to 11, wherein said linker comprises a polyethyleneimine backbone and hydrazide groups bonded to said backbone.

13. The method of claim 12, wherein said linker has a molecular weight of about 2000 to about 20000 Dalton.

14. The method of any one of claims 1 to 13, wherein said cells comprise an aldehyde group.

15. The method of claim 14, wherein said cells comprise cells that have been modified to form aldehyde groups on surfaces of said modified cells.

16. The method of any one of claims 1 to 15, wherein said cells comprise HepG2 cells or rat bone marrow stem cells.

17. The method of claim 3 or claim 4, wherein said flow of said fluid is actuated by applying a withdrawal force downstream of said distributed openings.

18. A cell culturing device, comprising:

a conduit;

a fluid flowing in said conduit, said fluid comprising cells, a transient linker and a cell culture medium, said linker facilitating initial attachment of adjacent cells to form a cell aggregate; and

a cage in said conduit for retaining aggregated cells formed in said fluid, said cage defining distributed openings that allow said fluid to flow through.

19. The device of claim 18, wherein said cage comprises a plurality of projections, arranged in a substantially U-shaped pattern.

20. The device of claim 19, wherein said projections comprise micro-pillars.

21. The device of claim 19 or claim 20, wherein said conduit has a bottom and opposing side walls extending from said bottom, said projections extending from said bottom.

22. The device of any one of claims 18 to 21, wherein said cells in said fluid have a density of about 5 to about 6 million cells/ml and said transient linker in said fluid has a concentration of about 6 to about 8 μM.

23. The device of any one of claims 18 to 22, wherein said cells are suspended in said fluid.

24. The device of any one of claims 18 to 23, wherein said linker is dissolved in said fluid.

25. The device of any one of claims 18 to 24, wherein said linker comprises a polyethyleneimine backbone and hydrazide groups bonded to said backbone.

26. The device of claim 25, wherein said linker has a molecular weight of about 2000 to about 20000 Dalton.

27. The device of any one of claims 18 to 26, wherein said cells comprise an aldehyde group.

28. The device of claim 27, wherein said cells comprise cells that have been modified to form aldehyde groups on surfaces of said modified cells.

29. The device of any one of claims 18 to 28, wherein said cells comprise HepG2 cells or rat bone marrow stem cells.

30. The device of any one of claims 18 to 29, wherein said fluid is actuated by a withdrawal force applied downstream of said distributed openings.