

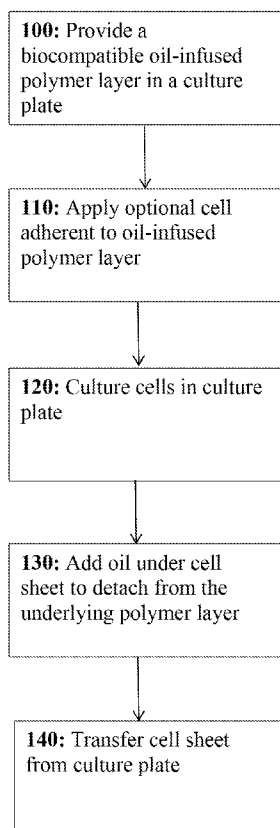


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(54) Title: DYNAMIC IMMISCIBLE LIQUID INTERFACES FOR CELL SHEET DETACHMENT

FIG. 1



(57) Abstract: A system for the growth and detachment of cells includes a biocompatible liquid- infused substrate adapted for culturing cells, wherein the liquid absorbed within the substrate is in an amount sufficient to form a slippery lubricating layer on a surface of the liquid-infused substrate. The system can include a cell adhesion agent deposited on the liquid-infused surface.





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DYNAMIC IMMISCIBLE LIQUID INTERFACES FOR CELL SHEET**DETACHMENT****RELATED APPLICATIONS**

[0001] This application claims the benefit of priority under 35 U.S.C §119(e) to co-pending United States application Ser. No. 62/158,287, filed May 7, 2015, the contents of which are incorporated in their entirety by reference.

INCORPORATION BY REFERENCE

[0002] All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described herein.

**STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR
DEVELOPMENT**

[0003] The present invention was made with United States government support under DARPA Grant No. N666001-11-1-4180 awarded by the Department of Defense and The United States government may have certain rights in this invention.

BACKGROUND

[0004] This disclosure relates to cell sheets useful in tissue engineering.

[0005] In recent years tissue engineering has seen much progress and has grown into a large and multidisciplinary field. It aims to restore, replace or regenerate defective tissues using a combination of cells, engineered scaffolds and growth stimulators. The most common methods for tissue engineering currently fall into four broad categories: pre-made porous scaffolds, decellularized extracellular matrix, cell sheets with secreted extracellular matrix (ECM) and cells encapsulated in self-assembled hydrogels.

[0006] Cell sheet engineering is a powerful and versatile technology with promise in a variety of applications. Sheets of mesenchymal stem cells (MSCs) in particular have been

successfully used for multiple applications including the repair of scarred myocardium after myocardial infarction, the rebuilding of cartilage, and the enhancement of healing in critical-sized femoral defects. Although MSCs are among the most popular cell types for cell-based therapies due to their multilineage differentiation potential into osteogenic, chondrogenic, or adipogenic cells, cell sheet engineering with other types of cells, including hepatocytes, epithelial cells, and myocardial cells, has also demonstrated the power and versatility of this technology.

[0007] The approach of using cell sheets with a secreted ECM is particularly useful for tissues with a high cell density such as those present in the heart and liver. It is a versatile technology that has been used with ligament cells hepatocytes, epithelial cells, myocardial cells, mesenchymal stem cells and many other types. Cells are grown to confluence such that they excrete their own ECM which acts as the scaffold that holds the tissue together. Cell sheet engineering enables the retention of cell-to-cell junctions and the native ECM.

[0008] Currently, the most common method for cell sheet harvesting involves the use of a thermoresponsive system based on the thermoresponsive polymer poly(N-isopropylacrylamide) (pNIPAAm) developed in the Okano Lab. pNIPAAm undergoes a reversible transformation between expanded hydrophilic coils and compact hydrophobic globules about its lower critical solution temperature (LCST) of 32°C. pNIPAAm grafted surfaces are hydrophobic above the LCST, at which points cells adhere and proliferate. Lowering the temperature below the LCST makes the surface hydrophilic, which causes the cultured cells to detach from the surface. Although this temperature-responsive cell sheet release has been proven effective across a wide range of applications, it is by nature subject to several limitations. First, the time to detach a single cell sheet from the current commercially-available thermo-responsive cell sheet surfaces can be 40 min or more, making it incompatible with high-throughput applications. Second, the need for temperature changes

to release the cells from the surface may change the gene expression or cell function in some more sensitive cell lines. Finally, creating pNIPAAm-coated surfaces for intact cell-sheet release requires electron-beam or vapor-phase polymerization equipment and facilities, which are not very common in biological labs. While pre-coated thermo-responsive surfaces are commercially available (e.g., UpCell), these materials can be prohibitively expensive in the quantities necessary to optimize cell-sheet release with a new cell line or for a new application. Other stimuli-responsive surfaces for growing and detaching cell sheets have also been explored, including electro-responsive and photo-responsive materials. Although improving in the areas of temperature changes and detachment time, these approaches also require highly specialized facilities, materials, or expertise to manufacture.

[0009] Mesenchymal stems cells (MSCs) are a population of stem cells present in adult tissue that can be isolated relatively easily and in quantities abundant enough for clinical use. The multilineage differentiation potential of MSCs into osteogenic, chondrogenic or adipogenic cells has made their use in therapeutic transplants and tissue engineering prevalent. Moreover, the use of mesenchymal stem cell sheets as an alternative to scaffolds seeded with MSCs or direct injection of MSCs to the site of injury has been demonstrated. MSC sheets cultured on and removed from thermoresponsive cultureware have shown to be able to differentiate into osteogenic cells to form bone grafts ex-vivo and revitalize allografts to treat bone defects. They have been directly injected into the site of myocardial infarctions in the form of cell sheet fragments as well as transplantation onto scarred myocardium to improve cardiac function and reverse wall thinning after coronary ligation. MSC sheets present a way to use the therapeutic potential of MSCs in a form that allows for greater cell retention post-transplant, greater viability, and secretion of native ECM and bioactive molecules.

SUMMARY

[0010] A surface for the growth and release of cell sheets is provided. The surface includes a biocompatible oil-infused polymer adapted for culturing cells, wherein the biocompatible oil is absorbed within the polymer in an amount sufficient to form a slippery lubricating layer on a surface of the liquid-swollen polymer. The surface is provided in a well-plate, tissue culture plastic, or other cell culture plate that contains a suitable culture medium for growth of cells on the surface.

[0011] In other aspects, a method for cell sheet growth and detachment is described. Cell sheets transferred using this method showed high viability and similar morphologies to controls grown on standard tissue culture surfaces. Growth and proliferation after transfer proceeded normally. This method of cell sheet growth and detachment may be useful for low-cost, flexible, and customizable production of cellular layers for tissue engineering. The method can be used for the formation of protein layers, e.g., protein free-standing films, as well as other biomolecule layers.

[0012] In one aspect, a system for the growth and detachment of cells includes a biocompatible water immiscible-liquid-infused substrate adapted for culturing cells, wherein the water immiscible-liquid absorbed within the substrate is in an amount sufficient to form a slippery lubricating layer on a surface of the water immiscible-liquid-infused substrate.

[0013] In one or more embodiments, the system further includes a cell adhesion agent deposited on the water immiscible-liquid-infused substrate.

[0014] In any of the preceding embodiments, the cell adhesion agent includes an extracellular matrix protein.

[0015] In any of the preceding embodiments, the substrate includes a polymer, or the substrate includes a porous solid.

[0016] In any of the preceding embodiments, the water immiscible-liquid includes an oil.

[0017] In any of the preceding embodiments, the system further includes a culture plate for holding the biocompatible water immiscible-liquid-infused substrate.

[0018] In any of the preceding embodiments, the biocompatible water immiscible-liquid-infused substrate coats the culture plate.

[0019] In any of the preceding embodiments, the culture plate includes a well-plate or tissue culture plastic.

[0020] In any of the preceding embodiments, the system further includes a culture medium selected to support the growth of cells on the biocompatible water immiscible-liquid-infused substrate contained within the culture plate.

[0021] In any of the preceding embodiments, the substrate is tube-shaped.

[0022] In any of the preceding embodiments, the substrate is polydimethylsiloxane and the water immiscible-liquid is a silicone oil.

[0023] In any of the preceding embodiments, the water immiscible-liquid-infused substrate is in the shape of a scaffold for tissue engineering. The substrate can be formed with complex geometries and surface patterns. The substrate can be curved or it can be molded to form complex 3D sheets.

[0024] In another aspect, a kit for the growth and detachment of cells, proteins and other biomolecules includes a culture plate including at least one well; and a layer of a biocompatible water-immiscible-liquid-infused substrate adapted for culturing cells coating the at least one well, wherein the water immiscible-liquid absorbed within the polymer is in an amount sufficient to form a slippery lubricating layer on a surface of the liquid-infused substrate.

[0025] In any of the preceding embodiments, the kit further includes a cell adhesion agent deposited on the water immiscible-liquid-infused substrate.

[0026] In any of the preceding embodiments, the cell adhesion agent includes fibronectin.

[0027] In another aspect, a cell system includes a biocompatible water immiscible-liquid-infused substrate adapted for culturing cells, wherein the water-immiscible liquid absorbed within the substrate in an amount sufficient to form a slippery lubricating layer on a surface of the water immiscible-liquid-infused substrate; and a confluent or near-confluent layer of cells adhered to the surface of biocompatible water immiscible-liquid-infused substrate.

[0028] In one or more embodiments, the cell system further includes a layer of cell adhesion agent disposed between the substrate and the cells.

[0029] In any of the preceding embodiments, the substrate is tube-shaped.

[0030] In another aspect, a method of cell sheet growth and detachment includes growing a cell of interest on a biocompatible water immiscible-liquid-infused substrate to confluency or near confluency to form a cell sheet, the water-immiscible-liquid absorbed within the substrate in an amount sufficient to form a slippery lubricating layer on a surface of the water immiscible-liquid-infused substrate; once confluency or near-confluency is reached, introducing a volume of oil underneath the cell sheet causing the cell sheet to separate from the surface while remaining intact; and transferring the cell sheet onto a new surface. The excess oil can be introduced in a variety of ways, including addition by syringe or other fluid delivery system, or by compressing, stretching or bending the liquid-infused substrate to cause the substrate to exude oil. In one or more embodiments, the delivery can be automated.

[0031] In one or more embodiments, cells are selected from the group consisting of ligament cells hepatocytes, epithelial cells, myocardial cells, mesenchymal stem cells.

[0032] In any of the preceding embodiments, the water immiscible-liquid-infused substrate includes an elastomeric polymer swollen with an oil.

[0033] In any of the preceding embodiments, excess oil is removed from the polymer surface.

[0034] In any of the preceding embodiments, the polymer is polydimethyl siloxane and the oil is a silicone oil.

[0035] In any of the preceding embodiments, the cell adherent agent is a extracellular matrix protein.

[0036] In any of the preceding embodiments, the cells are grown to at least 75% confluence, or at least 80% confluence, or at least 85% confluence, or at least 90% confluence.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] The invention is described with reference to the following figures, which are presented for the purpose of illustration only and are not intended to be limiting.

[0038] In the Drawings:

[0039] **Figure 1** is a process flow diagram illustrating a method of cell sheet growth and detachment, according to one or more embodiments.

[0040] **Figure 2A** is a schematic of the well-plate surfaces onto which cells were cultured for confluence experiments, including a well-plate with a liquid-infused polymer layer, with (center) and without (upper) a fibronectin coating layer and a control (lower) with no polymer layer.

[0041] **Figure 2B** shows representative images corresponding to cell growth on the well-plate surface shown in Figure 2A, including magnified views (right) of selected regions; the cells are stained with CV for easier visualization. Light microscopy images of the cell sheets from the FN-coated infused PDMS substrate and the TCPS control substrate (right). Scale bar is 50 μ m.

[0042] **Figure 2C** is a plot showing normalized biofilm density of cells grown on infused PDMS with no fibronectin coating and with a fibronectin coating. A TCPS control is

included for comparison. There is no significant difference between the densities of cells grown on FN-coated, infused PDMS and TCPS ($P = 0.907$).

[0043] **Figure 3A** is a plot showing normalized biofilm density of cells grown on infused PDMS with increasing concentration of FN coating the wells for 2, 3, and 4 days. The density was found to be highest after 4 days for wells coated with either 0.52 or 1.04 μg of FN.

[0044] **Figure 3B** is images of the surfaces used for analysis reported in Figure 3A. The cells are stained with CV for easier visualization. The scale bar is 1 cm.

[0045] **Figure 4A** is a schematic description of the peeling of the cell sheet of the surface: (i) silicone oil is added to the surface of the infused PDMS in an area of relatively low cell density in order to get the liquid underneath the cells. (ii) More silicone oil is added, creating a small pool. (iii) The pool of silicone oil is moved around the well plate, delaminating the cell sheet from the surface as it travels.

[0046] **Figure 4B** shows time lapse images of a pool of silicone oil moving around a well plate. Heavy dotted lines indicate the edge of the pool of silicone oil as it moves. A lighter dotted lines indicate the edges of the cell sheet that has been lifted off. The scale bar is 1 cm.

[0047] **Figure 5** shows time-lapse images showing complete, intact cell sheet transfer from the 6-well plate to a petri dish. The cells are stained with crystal violet for easier visualization. 1) The intact cell sheet is first completely delaminated from liquid-infused polymer substrate. 2) A piece of filter paper is placed onto cell sheet. 3) The filter paper with the attached cell sheet is removed from the well. 4) The intact cell sheet on the filter paper can now be transferred. 5) The cell sheet is placed in a petri dish, and the filter paper is removed. 6) The intact cell sheet is now ready for further processing. The scale bar is 1 cm.

[0048] **Figure 6A** are confocal images of a transferred cell sheet 2 h after transfer stained for FN at the level of the cell-surface interface. There is a large amount of FN present, indicating the transfer of the CM with the cell sheet. Images of the well post-transfer show

little to no remaining FN after the transfer of the sheet. A FN layer with no cells was also transferred from the infused PDMS, suggesting that the removal mechanism is dominated by the protein/oil interaction rather than by cellular metabolism. Scale bar, 200 μm .

[0049] **Figure 6B** are confocal images of a transferred cell sheet and controls stained for live and dead cells. The percent live cells in the transferred sheet was found to be similar to the values calculated for untreated PDMS and TCPS. Scale bar, 200 μm .

[0050] **Figure 6C** are confocal images of a transferred cell sheet stained for f-actin (green), fibronectin (FN, red) and nuclei (blue) at the level of the cells. Cells grown on TCPS and uninfused PDMS with FN shown for comparison. The morphology and arrangement of the cells appears similar between the sheet and the controls. Scale bar, 50 μm .

[0051] **Figure 7A** shows light microscopy images of the same region of a cell sheet grown on infused PDMS and transferred to TCPS immediately after transfer, 24 h after transfer with a change of medium, and after 48 h with another change of medium. The silicone oil drops (indicated by arrows) that are present are progressively washed away, and the sheet shows normal growth and proliferation. Scale bar, 1 mm. **Figure 7B** shows images for a cell sheet that has been loosened, but not transferred, using dyed silicone oil (left image). The dyed oil has coated the substrate and the edges of the cell sheet. Small groups of transferred cells show a coating of the dyed oil (center image), however the dye is washed away with a change of the medium (right image). Scale bar, 200 μm .

[0052] **Figure 8** is a plot of elastic moduli of infused PDMS made with different cross-linker.

[0053] **Figures 9A** and **9B** show images of a cells sheet before (9A) and after (9B) transfer. Scale bar, 50 μm .

DETAILED DESCRIPTION

[0054] Tissue engineering using whole, intact cell sheets has shown promise in many cell-based therapies. Improvements in cell sheet growth and detachment are described. In one or more embodiments of the cell-sheet detachment methodologies described herein, cells growing on the surface are able to form strong attachments with each other, as well as attachments with the surface that are sufficiently strong to allow normal growth and proliferation. At the desired time of sheet release, the surface switches from sticky to non-sticky, reducing cell sheet/substrate attachment strength and thus facilitating the lift-off of an uncompromised cell construct. The invention is described with reference to the growth and release of cell sheets, however, it is contemplated that the release method can be employed with other biomolecule layers, such as protein layers.

[0055] In one aspect, a substrate having a liquid layer disposed on the surface is used for cell growth. The cell culturing system includes a well-plate, tissue culture plastic, or other cell culture plate containing the liquid-coated substrate. The liquid coated substrate can be a flat surface that is treated to hold a liquid layer, a porous layer that can be infused with a liquid or a material that can be swollen with a liquid. The liquid coated substrate can be coated with a cell adhesion agent that promotes cell adhesion and growth. In use, cell of interest are grown on the liquid-coated surface to confluency or near confluency to form a cell sheet. As used herein, near confluency can include at least 75% confluence, or at least 80% confluence, or at least 85% confluence, or at least 90% confluence. Once confluency or near-confluency is reached, an excess amount of liquid (the same or similar to that of the liquid-coated substrate) is added underneath an edge of the cell sheet. The liquid addition process can be automated to accurately control the volume of liquid added to the system. The liquid loosens attachment of the cells to the culture plate and facilitates transfer of cultured cells.

[0056] In one aspect, oil-infused polymers are used as substrates for the growth and detachment of cell sheets. In one or more embodiments, bulk polymeric materials such as fluorogels or polydimethylsiloxanes (PDMS) are exposed to an excess amount of a chemically-matched oil. The polymers absorb the oil leaving a thin liquid layer on the material surface and holding a reservoir of the oil in the polymer bulk, which can diffuse to the interface and replenish the surface liquid layer as it becomes depleted. The liquid can be released from the bulk of the polymer by stretching or bending. These materials have proven highly effective at resisting bacterial adhesion under both static and flow conditions.

[0057] A cell culturing system includes a well-plate, tissue culture plastic, or other cell culture plate containing an oil-infused polymer layer. An oil-infused polymer includes a cross-linked polymer (e.g., such as a rubber or elastomer) that is solvated with a liquid having a chemical affinity for that polymer material. The oil-infused polymer can appear dry to the touch, but the molecularly porous polymer network is capable of being filled by a liquid, such as an oil, that can be retained in the network. The absorbed liquid in the polymer acts as a reservoir to maintain an equilibrium of the liquid layer on the polymer surface.

[0058] In one aspect, the substrate can be any substrate capable of infusing a water – immiscible liquid. The substrate can be, for example, a porous substrate or a substrate capable of swelling to absorb the liquid. In one or more embodiments, the substrate is a polymer and the liquid is an oil, e.g., a hydrocarbon or silicone oil. Oil-infused polymers can be formed by combining oils and polymers such that the polymer absorbs the liquids and forms a lubricating layer on a surface of the polymers (referred to herein also as “self-lubricating polymers”). Under selected conditions, the oil-infused polymer layer can exhibit low slip, anti-adhesion properties that are useful in the detachment and transfer of cell sheets.

[0059] In some embodiments, the oil-infused polymer layer is coated with a cell adhesion agent that promotes cell adhesion and growth. Due to the slippery nature of the oil-infused

polymers, the cells can have a tendency to form clumps as they grow, as they have better adhesion to each other than to the surface. To overcome this, cell adhesion agents can be applied to the polymer layer before cell seeding. These complexes are deposited onto the surface prior to cell seeding and allow the cells to adhere and proliferate on a surface.

Suitable cell adhesion agents include bioactive molecules such as ligands, hormones, enzymes, natural growth factors or synthetic cell behavior regulators, that are known in the art to promote cell adhesion. In one or more embodiments, the cell adhesion agent can be fibronectin, collagen or other extracellular matrix protein.

[0060] Particularly suitable exemplary cell adhesion agents include fibronectin, collagen, or other extra-cellular matrix protein that are suitable for use as a cell adherent. Fibronectin is an abundant ECM protein with adhesion promoting properties. The integrin receptors present in the cell bind to the RGD peptide sequence in fibronectin and allow for the generation of cytoskeleton tension which results in the formation of focal adhesion complexes. These complexes allow the cells to adhere and proliferate on a surface.

[0061] In some embodiments, the infused polymer is formed as a sheet and the cells grow on its surface as a cell sheet. In other embodiments, the infused polymer is formed, e.g., molded, cast, cut or otherwise shaped, into a pre-selected shape. The polymer can be shaped before or after infusion with the swelling liquid and/or application of the cell adherent agent. The cells grow on the shaped polymer and take on the form factor of the polymer. In such instances, the infused polymer acts as a template or scaffold to direct the growth of the cells. In other embodiments, the polymer sheet is used as a layer in a cell culturing device, such as a well plate or other culture plate. In other embodiments, surface patterns can be included on the polymer surface.

[0062] In another aspect, a method of cell sheet growth and detachment is described. Briefly, the method includes growing the cell of interest on an oil-infused polymer surface to

confluency or near confluency to form a cell sheet. Once confluency or near-confluency is reached, an excess amount of oil (the same or similar to that of the oil-infused polymer) is added underneath an edge of the cell sheet. The liquid loosens attachment of the cells to the culture plate and facilitates transfer of cultured cells. The pool of oil created can be positioned underneath the cell sheet, causing it to separate from the surface while remaining intact. The sheet can be then transferred onto a new surface using filter paper. The method can be carried out at room temperature, and can be completed rapidly in a few minutes.

[0063] The invention can employ any cell population that is capable of proliferation in *in vitro* conditions. In particular, cells useful in tissue engineering are contemplated, such as ligament cells hepatocytes, epithelial cells, myocardial cells, mesenchymal stem cells, and the like. Suitable cells types for use in this invention include those which can grow on an artificial substrate coated with an adherent agent such as those described above. The cells or cell sheets should also be robust enough to withstand the slight change in underlying topography that comes with the introduction and movement of a pool of excess silicone oil under the adherent agent/cellular layer, as well as the potential stress of transfer after the layer is released.

[0064] The method is described with reference to the process flow diagram in Figure 1.

[0065] In step 100, a biocompatible oil-infused polymer is provided. To accomplish this, a polymer sheet or layer is infused with a compatible oil. The disclosed self-lubricating polymer can be made from a broad range of polymers and lubricating liquids.

[0066] The polymer material can be chosen from a wide range of rubbers and elastomers, and other polymers, which can swell significantly in the presence of certain solvent lubricating liquids. In particular, the polymer can be rubber or elastomeric polymers, which are known to swell in the presence of an appropriate solvating liquid. In some embodiments, the polymer is a nonporous material. The polymer, e.g., an elastomer or rubber, is typically a

covalently cross-linked polymer. The polymer can be a simple single polymer or complex mixture of polymers, such as polymer blends or co-polymers and the like. The nature and degree of crosslinking can change the properties of the polymer. For example, cross-linking density can be used to control how much the polymer will swell (e.g., a lightly cross-linked polymer may swell more than a highly cross-linked polymer). In addition to controlling the degree of swelling, the crosslink density also tunes the modulus of elasticity of the polymer, which can be adjusted to match the polymer modulus to different biologically relevant moduli for different cells, e.g., osteoblasts, myoblasts, adipocytes, etc.). The modulus can be selected, for example, to assist in cell differentiation. In other embodiments, magnetic nanoparticles or other materials can be included in the polymer, which can be used to externally manipulate the polymer shape.

[0067] Exemplary polymers include natural and synthetic elastomers such as Ethylene Propylene Diene Monomer (EPDM, a terpolymer of ethylene, propylene and a diene component), natural and synthetic rubbers, fluoroelastomers, perfluoroelastomers, polyether block amides (PEBA), chlorosulfonated polyethylene (CSM), ethylene-vinyl acetates (EVA), polybutadienes, polyether urethanes, fluorinated hydrocarbon (Viton), silicones, fluorosilicones, polyurethanes, polydimethylsiloxanes, and vinyl methyl silicones.

Particularly suitable exemplary polymers include siloxanes, such as polydimethylsiloxane (PDMS), fluorogels, or crosslinked fluorinated polymers. PDMS is an attractive polymer selection as it is chemically inert and non-toxic to mammalian cells and is already widely used in biomedical devices, medical implants, and microfluidic cell culturing.

[0068] The swelling or lubricating liquid can be selected from a number of different biocompatible liquids. Suitable lubricants can be chosen from a wide range of liquids (solvents) which have an affinity for the selected polymer such that the liquid causes the polymer to swell and absorb the liquid as described above. The chemical affinity creates a

solvent effect that causes the polymer to absorb an amount of the liquid and swell. A cross-linked polymer is capable of increasing its volume up to several folds by absorbing large amounts of solvent. The swollen polymer network is held together by molecular strands that are connected by chemical bonds (cross-links). A cross-linked polymer is capable of increasing its volume several folds by absorbing large amounts of solvent. To swell the polymer, the enthalpy of mixing between the polymer and the lubricating liquid should be sufficiently low so that they mix readily with each other when mixed together, and/or undergo energetically favorable chemical interactions between each other.

[0069] In one or more embodiments, the lubricant is selected from the group consisting of fluorinated lubricants (liquids or oils), silicones, mineral oil, plant oil, water (or aqueous solutions including physiologically compatible solutions), ionic liquids, polyalpha-olefins (PAO), synthetic esters, polyalkylene glycols (PAG), phosphate esters, alkylated naphthalenes (AN) and silicate esters. Generally, the lubricating liquid is matched chemically with the polymer that it is solvating. For example, when the polymer is a hydrophobic polymer such as polydimethylsiloxane (PDMS), the lubricating liquid can be a hydrophobic liquid such as silicone oil, hydrocarbons, and/or the like. In other embodiments, a polydimethylsiloxane (PDMS) elastomer (e.g., such as methyl, hydroxyl, or hydride-terminated PDMS) can be used with a silicone oil. Hydride-terminated PDMS has been demonstrated to show good swelling with a range of lubricating liquids. Hydroxyl-terminated silicone oil in PDMS is also another type of swellable polymer providing oleophobic/hydrophilic surface. Other oils, such as perfluoropolyethers can be used to infuse the fluorinated polymers. Additional information on oil-infused polymers can be found in WO 2014/012080, which is incorporated in its entirety by reference.

[0070] In one or more embodiments, the polymer is PDMS , as this material is chemically inert and non-toxic to mammalian cells and is already widely used in biomedical devices, medical implants, and microfluidic cell culturing, and the oil is a silicone oil.

[0071] In one or more embodiment, the polymer precursor is applied to a culture plate or well and cured. As noted above, it is also possible to shape the polymer into more complex articles or shapes. The cured polymer is then allowed to swell (from several hours to several days) in the swelling liquid, optionally under elevated temperatures, to infuse the polymer with oil. The excess oil is then removed from the polymer sheet; the polymer sheet is rinsed with water and excess oil is removed. Removal of excess oil helps to reduce pooling of oil on the oil-infused polymer surface that can impair attachment of cells to its surface.

[0072] In step 110, a cell adhesion agent is applied to the oil-infused polymer layer (as used herein cell adherent agent and cell adhesion agent can be used interchangeably). The cell adhesion agent is selected to increase adhesion properties and provide other signals needed for growth and differentiation. Due to the slippery nature of infused PDMS, the cells have a tendency to form clumps as they have better adhesion to each other than to the surface. To overcome this, a cell adhesion agent can be deposited onto the surface prior to cell seeding. It was found that this process could produce a uniformly confluent cellular layer on the oil-infused polymer layer, while surfaces without a cell adhesion layer showed less growth and proliferation.

[0073] Deposition can be accomplished by incubating the polymer layer in a solution containing the agent, by spraying the agent over the surface, by dropping the agent in a solvent onto the surface and allowing the solvent to evaporate away, or by stamping the agent onto the surface. By initially removing the surface oil layer and depositing a layer of cell adhesion agent in its place, it is possible for cells to attach to the polymer substrate. In one or more embodiments, the cell adhesion agent is applied directly to the polymer.

[0074] In step 120, cells are introduced onto the cell plate and cultured. The polymer is preferentially swollen by the lubricating liquid rather than the cell culture medium, and therefore the lubricating layer is not displaced by the cell culture medium. Cells can be grown using suitable growth medium and growth conditions, as are generally known in the art. Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37 °C, 5% CO₂ for mammalian cells) in a cell incubator. Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can be readily determined. According to one or more embodiments, a high degree of cell growth and proliferation leading to confluent cell monolayers can be achieved. In one or more embodiments, the cells are grown to greater than 75% of confluence, or greater than 85% confluence, or greater than 90% confluence or cells that are 100% confluent.

[0075] In step 130, the cell sheet is detached from the underlying polymer layer. The cell sheet and its secreted ECM can be detached with the addition of an excess of the oil used to infuse the polymer. Alternatively, a different oil that is compatible with the polymer, but immiscible with the culture medium, can be used. The oil is introduced between the surface of the infused PDMS and the cell sheet layer. Alternatively, the polymer sheet can be stretched or bent to exude oil from its reservoir. In other embodiments, magnetic particles embedded in the polymer sheet can be activated by application of a magnetic field to release oil from the sheet or to facilitate movement of the oil over the sheet surface. The detachment method is illustrated in Figure 4A, in which a small amount of silicone oil is introduced under the cell sheet using a syringe. As noted previously, this process can be automated. In one embodiment, the oil droplet is added in an area of relatively low cell density, as this represents an area where liquid is easier to get under the cell sheet. As the oil droplet passes between the polymer layer and the cell sheet, the cells and the extracellular matrix, is gently lifted from the surface. The amount of oil can be small. It is not required that the entire

polymer layer be coated by additional oil since the cell sheet remains detached once lifted by the oil droplet from the surface. Although this process worked best for sheets that were not yet completely confluent, e.g., greater than 75% of confluence, or greater than 85% confluence, or up to 90% confluence, thicker sheets, e.g., 100% confluent cell sheets, could also be removed. Removal is aided by encouraging the separation with tweezers.

[0076] In step 140, the detached cell sheet layer can then be moved to a new surface, such as tissue culture polystyrene [TCPS]. Transfer can be accomplished by adsorption of the cell sheet onto filter paper, for example by pressing the filter paper on to the cells and aspirating out any excess fluid (e.g., culture medium). The filter paper can be transferred to a new culture surface for further culturing or storage. The transfer process is shown in Figure 5.

[0077] By way of example, oil-infused PDMS can be used to grow and release intact sheets of mouse mesenchymal stem cells. A thin layer of PDMS can be used to coat the bottom of a well plate, which is then infused with silicone oil to produce the oil-infused PDMS substrate. After removal of the excess silicone oil and application of a layer of fibronectin, mouse mesenchymal stem cells can be seeded on the surface.

[0078] The cells can be grown to confluency or near-confluency. The resultant cell sheet can be released from the surface by introducing an excess amount of silicone oil underneath the cell sheet. This excess oil slides underneath the cell sheet, releasing it from the surface. The cells sheet can then be transferred to a new surface using filter paper.

[0079] Immunofluorescent staining can be used to confirm the presence of fibronectin in the ECM of the transferred cell sheet as well as show the similarity in morphology and cytoskeleton structure of the cells before and after transfer. Immunostaining of the cell sheets post-transfer showed similar distributions of fibronectin and f-actin as cell sheet grown on tissue culture polystyrene. Live/dead staining showed viability after transfer to be nearly as high as cells grown on control tissue culture polystyrene surfaces. Staining for live cells

showed 97% viability for nearly-confluent sheets after transfer. After transfer, growth and proliferation of the cells proceeded normally. Although some oil droplets were present on the surface, these did not appear to hinder cell growth and could be washed away through normal changing of the growth medium.

[0080] Over the last few years, a growing body of literature has emerged on the use of immobilized liquid overlayers, such as the ones used here, to repel a range of materials including bacteria and various biomolecules. The concept is that organic, inorganic, or biological substances can settle on the liquid layer, but are easily removed by any disturbance to the system due to the liquid nature of the surface they sit on. According to one or more embodiments, immobilized liquid overlayers can be used not only for the repulsion of unwanted material in various antifouling applications, but can be highly beneficial in tissue engineering for cell culturing purpose. To achieve this, the slippery surface containing an immobilized liquid overlayers is coated with a cell adhesion layer that, e.g., denatures and forms a web on the surface of the liquid layer. To facilitate the deposition and attachment of the cell adhesion layer, the immobilized liquid overlayer can be initially thinned by an oil removal step immediately prior to the cell adhesion agent deposition. The thinness of the layer limits the mobility of any liquid molecules on the surface and allows the deposited layer to be stable enough for initial cell attachment and proliferation.

[0081] Over time, the oil overlayer gradually thickens due to the diffusion of the oil from the polymer bulk, providing more mobility to the surface liquid film that supports the cell adhesion layer and the increasingly dense cellular sheet. To fully release the cell construct from the substrate, an excess volume of a releasing liquid, e.g., a water immiscible oil such as silicone oil, is introduced in between the surface of the infused polymer and the cell adhesion layer. This thickens the oil layer further, creating an oil pool which is moved around the well, weakening the cell sheet-surface interactions and delaminating the cell sheet from the surface

as it travels. This allows the cell adhesion/cellular layer to float freely on the oil interface, and makes it possible to transport the entire sheet from the substrate simply using filter paper. This mechanism is further supported by the observations that a protein based (fibronectin) cell adhesion layer can be transferred directly without a cellular layer and that some oil is transferred with the cells and the fibronectin, suggesting the cells themselves are not an integral part of the removal mechanism. Moreover, no difference has been observed in the release of fixed cells versus living cells, and efficient sheet release was not possible on samples that were not fully infused, suggesting that the mechanism of removal relies primarily on the penetration of excess oil between the solid surface and the cell adhesion layer/cellular sheet, overcoming any attractive forces present between the two, and is not governed by the metabolic activity of the living cells and their interaction with the underlying substrate.

[0082] In one aspect, a new, simple, and low-cost approach to cell-sheet detachment surfaces which could prove useful in the further expansion of the tissue engineering field. Furthermore, the use of polymers such as PDMS as the substrate may open doors to the use of more complex geometries, the incorporation of patterns, or the tuning of the substrate elastic modulus. For example, due to the shapability and moldability of the starting polymer, it is possible to grow cells in a variety of shapes. For example, the oil-infused polymer can be in the shape of tubes and the resultant cell sheets (also in the shape of tubes) can be used to form blood vessels. In other embodiments, the polymer can be formed into the shape of an organ, such as heart cells grown onto a heart-shaped polymer substrate.

[0083] In one aspect, oil-infused polymers can be used as cell-sheet release surfaces. This approach offers many advantages compared to current state-of-the art cell-sheet-release technologies: the low cost of the materials necessary to fabricate these surfaces (approximately \$0.02/cm²), the easy benchtop fabrication, and the short time needed to

completely release the sheet from the surface. In addition, it is possible to form and/or pattern the substrates, and thus the cell sheets that grow on them, into any desired shape. For example, it is possible to use tube-shaped infused polymer templates for cell growth. The resultant cell sheets are also in the form of tubes. This is particularly useful for growth of endothelial cells which can be used for tissue engineering of blood vessels.

[0084] In other embodiments, the method is used for growth and transfer of single (unattached) cells or for small patches (non-confluent) of cell sheets. The ability to transfer single cells is advantageous for cells that are hard to grow, that do not flourish on adhered surfaces, or which are damaged in transport. The methods described herein can permit the growth and release of cells that are difficult to detach, lose viability due to surface protein degradation, or are unintentionally activated with traditional surface release mechanisms such as trypsinization. The method can also be used for the transfer of protein sheets, extracellular matrices, polysaccharides and other biomolecular constructs.

[0085] Such benefits may serve to facilitate the further development and use of cell-sheet-based therapies in medicine.

[0086] The invention is described with reference to the following examples, which are presented for the purpose of illustration only and are not intended to be limiting of the invention.

Preparation of infused polydimethylsiloxane (PDMS) well plates

[0087] Tissue culture polystyrene well plates were plasma-treated for 5 min at a power of 250 W and oxygen gas flow of 15 sccm in a plasma etching chamber (Plasma Etch, Inc. PE-200, Carson City, NV). PDMS was prepared with the Sylgard 184 silicone elastomer (Dow Corning Corporation, Midland, MI). The base and curing agent were combined in a 10:1 ratio and mixed in a Thinky planetary centrifugal mixer (Thinky Corporation ARE-310, Tokyo, Japan) at 2000 rpm for 1 min, then again at 2200 rpm for 1 min. Well plates were removed

from the plasma chamber and immediately filled with roughly 1 g of the PDMS mixture per well. The well plates were then placed inside a 70°C oven (VWR Signature Forced Air Safety Oven 52201-216, Radnor, PA) for 5 min to ensure proper bonding between the plasma-treated polystyrene and PDMS. After curing, the PDMS-coated well plates were degassed in a vacuum chamber (VWR Symphony Vacuum Oven 414004-582, Radnor, PA) for a minimum of 1 hour and cured at 70°C for at least 2 h. To infuse the PDMS coating, roughly 2mL of Momentive 14 10A silicone oil (an excess amount) was placed in the well for 48 h, enough time for the PDMS to become completely infused.

Removing Surface Oil and Fibronectin Deposition

[0088] After infusion, the excess silicone oil was removed from the surface to ensure deposition of a FN layer uniform and stable enough for the cells to attach and proliferate. First, the excess oil was aspirated out of the well plates and the surfaces were dried with a nitrogen gas gun. The sides of the wells were wiped with a lint-free tissue to remove any silicone oil residues. Silicone sponges (McMaster-Carr 86235K142 Robbinsville, NJ) were then placed inside the well plates for 2 h to absorb any surface silicone oil from the PDMS surface. Immediately before fibronectin deposition, the surfaces were again dried with a nitrogen gas gun and rinsed with 70% ethanol to sterilize.

[0089] Human plasma fibronectin (Millipore, FC0010, Billerica, MA) was used to coat the dried, infused PDMS well plates. PDMS well plates were incubated in 1.25µg/mL, 2.5µg/mL or 5.0µg/mL solutions of fibronectin in Hank's Balanced Salt Solution (HBSS) for 2 h at room temperature on a rocking platform. The positions of the plates on the platform were changed intermittently throughout the two hours. Well plates incubated in HBSS without any fibronectin acted as controls.

Cell Culture

[0090] D1 ORL UVA [D1] (ATCC® CRL-1242, Manassas, VA) mouse bone marrow mesenchymal stem cells (MSCs) were used for all experiments. D1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose formulation with pyruvate (Gibco, 11995) with the addition of 10% Fetal Bovine Serum (Gibco, 10437) and 1% Penicillin-Streptomycin (Gibco, 15140). Cells were incubated at 37°C with 5% CO₂. For all experiments, cells were seeded at 5×10^4 /mL. For this study, cells in passages 26-39 were used.

Cell Proliferation Assay

[0091] Cell proliferation was measured by staining the cells with Crystal Violet (CV) according to a modified version of a method developed by Gillies et al.³¹ The cells were washed twice with HBSS and then fixed with 1% glutaraldehyde (Alfa Aesar) in HBSS for 15 min at room temperature. The fixed cells were then washed with HBSS and stained with 0.1% CV in deionized, distilled (DI) water for 30 min. Well plates were rinsed with DI water at least 5 times to remove all residual CV. Well plates were photographed with Canon Rebel T4i (Canon). Image analysis was conducted with MATLAB to determine the density of the cell sheet in each well. Briefly, the images were first thresholded to determine the area of the cell sheet relative to the area of the well. Then the intensity values were used to generate a surface plot of the cell sheet area. Integrating under the surface plot gave the density of the cell sheet. This was normalized to a hypothetical cell sheet that was black (i.e. intensity value of 1) with the area equivalent to that of the well (i.e. 100% coverage). Thus a darker purple color gave a higher intensity value and hence a higher density value. One-way and two-way ANOVAs were conducted to determine significance among the various conditions. Statistics were carried out with IBM SPSS Statistics 22 (IBM).

Detachment of Cell Sheets

[0092] Approximately 850 μ L, or 90 μ L/cm², of filter-sterilized silicone oil (0.45 μ m pore filter; needles: BD Precision glide 21g (model 305165) or 23g (model 305143); syringe: 1mL NormJect Nonpyrogenic/nontoxic, 4010-200V0, Henke-Sass, Wolf, Tuttlingen, Germany), or 90 μ L/cm², was injected under the cells onto the PDMS. Care must be taken not to puncture through the PDMS layer, otherwise delamination of PDMS will begin (delamination from punctures is not immediately noticeable as it occurs over the course of 2-3 days). The silicone oil formed a pool underneath the media solution on the surface of the polymer. This bubble was slowly rolled around to peel off the cell sheet, which rested on the oil-media interface. In places where the cells were more strongly adhered, flat-tipped tweezers were used to guide the silicone oil under the cell sheet to lift it off.

[0093] The cell sheet was transferred to a new culture surface for further culturing and processing using filter paper. Briefly, the silicone oil bubble was aspirated out, and filter paper sheet. Media was aspirated out from above the filter paper so that the cell sheets would adhere to the filter paper through capillary force. The filter paper was then peeled off and transferred to the new culture surface (35mm petri dish) where fresh media was added to detach the cell sheet from the filter paper. The cell sheet detaches from the filter paper in less than 5 min, at which point it could be removed from the media. The new culture surface with the transferred cells was then placed into the incubator to allow the cells to continue growing and form strong attachment bonds to the new surface. To ensure that the cells had reattached sufficiently, at least 1 h was allowed for the cells to reattach before further processing was done.

Cell Sheet Viability

[0094] To determine the viability of the cell sheets after transfer, Calcein AM was used to detect intracellular esterase activity and Sytox Orange Nucleic Acid to detect membrane integrity. Transferred cells were washed with HBSS twice and then incubated in 2 μ M Calcein

AM (Molecular Probes, C3099) and 2uM Sytox Orange (Molecular Probes, S11368) in Serum-free DMEM at 37°C for 1 h. The stained cells were then washed twice with HBSS and imaged in Live Cell Imaging Buffer (Molecular Probes, A1429DJ). Cells growing on infused PDMS (state of cells before detachment) and cells growing on TCPS were also stained and imaged as controls. A Zeiss LSM 710 confocal microscope was used to image the cells using the preloaded excitation and emission wavelengths set for the Calcein AM and Sytox Orange dyes. Image analysis (n=3) was used to calculate the coverage of the two dyes relative to the total dyed cell sheet area to quantify the viability. Image analysis was carried out with Zen image acquisition software (Zeiss) and MATLAB (Mathworks).

[0095] Experiments using dyed silicone oil to determine the localization of the oil after transfer were performed using a 1:1 mixture of silicone oil and difluoro{2-[1-(3,5-dimethyl-2H-pyrrol-2-ylidene-N) ethyl]-3,5-dimethyl-1H-pyrrolato-N}boron dye (Sigma-Aldrich). For these experiments we used small groups of individual cells, as opposed to an entire sheet, to ensure the maximum possible contact of the oil with the cells and thus the maximum possible coating or uptake of the oil by the cells. Thus these cells were not as securely attached to the surface and were more easily washed away. To ensure that any apparent removal of the dye was due to the washing rather than bleaching of the fluorophores, cells were released from the surface and immediately imaged, then washed and imaged again.

Immunofluorescent staining

[0096] Cell sheets were washed with HBSS twice and then fixed with 1% glutaraldehyde in HBSS for 20 min at 4°C. Fixed cells were then washed with HBSS and permeabilized with 0.5% Triton X-100 in HBSS for 10 min and subsequently blocked with stain buffer containing 0.2% BSA in PBS (Pharmingen) for 30 min at room temperature. Cells were then immunostained with a 1:200 dilution (1µg/ml) of rabbit polyclonal anti-fibronectin antibody (SantaCruz Biotechnology, sc-9068). Cell sheets were washed with BSA buffer twice before

reacting with a 1:2000 dilution (1 µg/ml) of Alexa Fluor 633 conjugated goat anti-rabbit secondary antibody for 1 h at 4°C. Cells were also co-stained with a 1:1500 dilution of Alexa Fluor 546 conjugated phalloidin to stain f-actin and 1 µg/ml Hoechst 33342 nuclear stain. Cells were washed twice in BSA buffer before imaging under liquid. Cell sheets before detachment and confluent cell layers grown on TCPS covered with fibronectin were also immunostained and imaged as controls. Cells were imaged with a Zeiss LSM 710 confocal microscope using the preloaded excitation and emission wavelengths set for Alexa Fluor 633, Alexa Fluor 546 and Hoechst dyes. A confocal z-stack of the cells was taken in each well (n=3). The base of the cell sheet (i.e. the imaging plane that showed the basal layer of the cell sheet in focus) was used for image analysis to calculate the amount of fibronectin per nuclei and f-actin per nuclei to quantify ECM and cell spreading morphologies. Image analysis was carried out with Zen image acquisition software (Zeiss) and MATLAB (Mathworks).

Preparation of PDMS substrates with variable elastic modulus

[0097] PDMS (Dow Sylgard 184 polydimethylsiloxane) samples for elastic modulus testing were made by combining polymer base to curing agent at 5:1, 10:1, 20:1 and 30:1 ratios. After curing, the samples were immersed in 10 cSt silicone oil for a minimum of one week to allow for maximum absorption. Control samples were not placed in silicone oil. The samples were placed between silicone sponges for approximately 10 min to remove any excess surface oil. The Young's moduli of the samples were determined using the Agilent Nano Indenter G200 with a (100 µm diameter) diamond flat punch. During each test, the flat punch contacted the surface of the sample and oscillated to determine the complex moduli of the samples. The complex modulus was determined at four points on each sample. Young's modulus was then calculated using a Poisson's ratio of 0.5.

Visualization of the replenishment of the oil overlayer

[0098] The surface of the infused PDMS samples over time after removal of the excess oil with silicone sponges were imaged using phase-contrast optical microscope (AX10, Carl Zeiss AG, Oberkochen, Germany) at low magnification. Silicone sponges were placed in contact with the sample surfaces for 24 h, then removed and immediately imaged for a $t=0$ measurement. Images were taken every hour thereafter for up to 48 hours

RESULTS

[0099] To prepare the oil-infused surfaces for cell growth, we first coated the bottom of plasma treated 6-well TCPS plates with 1.08 g (± 0.05 g) of uncured polydimethylsiloxane (PDMS) polymer, resulting in a 1.2 mm-thick coating covering the 35 mm well after curing. Trimethoxyterminated silicone oil (10 cSt, ~ 2 mL) was then introduced on top of the PDMS coating and allowed to infiltrate the polymer for 48 h to ensure full uptake. To ensure that complete infusion of the PDMS layer had occurred after 48 hours, the change in thickness of a 2 mm-thick slab of PDMS undergoing infusion was measured over time. The thickness was chosen to be twice as thick as the PDMS coating of the wells of the plate used to grow the cells (~ 1 mm) to account for the fact that the slabs were infused from both the top and the bottom, rather than just the top (as would be the case for the coating in the well plate). The slabs were immersed in excess 10cSt silicone oil, and removed and photographed from the side periodically to determine thickness change. It was found through fitting with a modified Langmuir isotherm function that the thickness of the slab stopped increasing after 21.7 h of exposure to the excess silicone oil. On average, the polymer coatings took up 0.49 mL (± 0.02 mL) of the oil, resulting in a swelling ratio of 1.42 (± 0.01) by mass. At the 10:1 base to crosslinker ratio used for cell growth, a slight decrease from 2.12 (± 0.09) to 1.47 (± 0.05) MPa was observed. Measurement of the elastic modulus of the infused polymer was also performed via nanoindentation and are shown in Figure 8. Figure 8 shows the values for the elastic moduli as determined by the indentation method were 2.74 (± 0.07) MPa, 2.12 (± 0.09)

MPa, 0.89 (± 0.01) MPa and 0.43 (± 0.03) MPa for the 5:1, 10:1, 20:1 and 30:1 controls, respectively. Young's moduli for the 5:1, 10:1, 20:1 and 30:1 infused PDMS samples were 2.50 (± 0.06) MPa, 1.47 (± 0.05) MPa, 0.37 (± 0.01) MPa and 0.12 (± 0.03) MPa, respectively. All cross-linker ratios demonstrated a decrease in Young's modulus following infusion with 10cSt silicone oil. 5:1 samples were found to have the smallest decrease in elastic moduli with an average difference of 0.24 MPa between its non-infused and infused state. 10:1, 20:1 and 30:1 samples have an average decrease in elastic moduli of 0.65 MPa, 0.52 MPa and 0.31 MPa, respectively. No delamination of the infused PDMS from the TCPS well surface occurred. Before cells were introduced to the system, excess surface oil was removed by washing the surfaces with water, blotting with a silicone sponge, and finally washing with ethanol.

[0100] To create a more favorable environment for growth and proliferation, we coated our surfaces with human plasma fibronectin (FN) prior to cell seeding. In this particular example, a concentration of FN was 1.25 $\mu\text{g/mL}$ or approximately 0.52 $\mu\text{g/cm}^2$ of infused polymer surface. The effect of the FN concentration on cell density is shown in Figures 3A and 3B. To test the effect of fibronectin concentration on cell sheet growth in this system, cells were grown on infused PDMS surfaces following incubation in 0, 1.25, 2.5 or 5.0 $\mu\text{g/mL}$ fibronectin solutions (0, 0.52, 1.04, or 2.08 $\mu\text{g/cm}^2$) for 2, 3 or 4 days. After the specified growth time, cells were fixed, stained, photographed and peeled off for further processing. As expected, wells with no fibronectin showed very little cell proliferation. Interestingly, wells incubated in 2.08 $\mu\text{g/cm}^2$ fibronectin also had significantly less cell proliferation than 0.52 $\mu\text{g/cm}^2$ and 1.04 $\mu\text{g/cm}^2$ across all 3 days ($P < 0.05$). This may be attributed to clumping and aggregation of the fibronectin protein, preventing the RGD binding site from being exposed to the cells.²⁶ As there was no significant difference in cell

proliferation between wells incubated in $0.52 \mu\text{g}/\text{cm}^2$ and $1.04 \mu\text{g}/\text{cm}^2$ fibronectin ($P = 0.567$), $0.52 \mu\text{g}/\text{cm}^2$ fibronectin solutions were used for all other experiments.

[0101] Optimization of the incubation time for cell sheet growth in this system was also investigated. Each of the incubation times tested (2, 3, and 4 days) had its own advantages and disadvantages and incubation time selection was dependent on the degree of confluence that was desired. Cell sheets harvested after 2 days growth readily detached with just the advancement of the silicone oil interface, but produced less confluent sheets that required more careful handling. Cell sheets harvested after 4 days growth required the use of tweezers to aid in detachment but produced much more robust confluent sheets that were easy to handle and transfer. For cell sheets harvested after 3 days growth, some areas detached with the advancement of the interface, whereas others needed the help of tweezers. These sheets were much more robust and easier to handle compared to those harvested after only 2 days growth. In all cases, cell sheets could be released from the surface within minutes, with an average release time of ~ 3.5 min.

[0102] Cell growth on FN-coated and uncoated infused PDMS was compared to unmodified TCPS controls and FN-coated non-infused PDMS substrates, shown schematically in Figure 2A. FN-coated infused PDMS showed a ~ 20 -fold increase in cell proliferation based on the cell sheet density compared to uncoated surfaces. Figure 2B are images of cell growth. There was no significant difference ($P = 0.907$) between the densities of cells grown on fibronectin-coated infused or noninfused PDMS and those grown on the TCPS control surface, as shown in bar plot in Figure 2C.

[0103] Once the cell sheet had grown to confluence or near-confluence, the cells were loosened by injecting an excess volume (approximately $850 \mu\text{l}$ total or $90 \mu\text{l}/\text{cm}^2$) of silicone oil between the FN layer/cell sheet and the infused surface. The newly-created oil pool was then gently maneuvered around the well by tilting the plate to separate the cell sheet from the

surface. Figure 4A is a stepwise schematic of the process and Figure 4B shows a series of time lapsed photos demonstrating the cell sheet detachment. (Using this simple procedure, an intact cell sheet could be released within minutes (5 ± 1.9 min on average)). As a control, this removal method was also attempted with cells grown on non-infused, FN-coated PDMS, as well as partially-infused PDMS (infused for only 4 h). It was found that in these cases the cell sheet could not be detached using added silicone oil, even when encouraging detachment with tweezers or a cell scraper, and simply shredded upon attempted removal. After the release from the FN-coated infused PDMS substrate, the cell sheet could be easily transferred to a new culture surface, for example using filter paper. Figure 5 is a series of time lapsed images showing the transfer of a cell sheet from the well plate. A filter paper circle was gently placed in the well over top of the cell sheet. Media was aspirated from above the filter paper so that the cell sheet adhered to the filter paper through capillary force. The filter paper was then peeled off and transferred to the new culture surface where fresh media was added to detach the cell sheet.

[0104] Previous work on infused PDMS and other substrates for immobilized liquid layers have established that proteins and microorganisms are easily removed from these surfaces due to the inherent mobility of the liquid itself. To confirm that there was still an oil layer present after removing the excess before FN deposition, we visualized the surfaces over time immediately following oil removal. The results showed a replenishment of the surface oil over 48 h, supporting the hypothesis that the FN/cellular layer was sitting on top of an immobilized liquid layer when it was detached from the growth surface. An infused PDMS surface was visualized over time via brightfield microscopy after removing the excess oil with silicone sponges. The images showed a slow replenishment of the oil on the surface over 48 hours, as indicated by the gradual disappearance of surface defects. This supported the hypothesis that the FN/cellular layer was sitting upon an immobilized liquid layer which,

upon addition of excess oil, thickened and released the sheet from the surface. The more direct evidence for this mechanism is the removal of the fibronectin extracellular matrix (ECM) layer together with the cell sheet. Images of the cell sheets 2 h after transfer showed a continuous FN layer underneath the cell sheet (Figure 6A, left), while images of the well from which the sheet was transferred showed little to no FN remaining (Figure 6A, middle). Even in the absence of the cells, the FN layer alone is successfully delaminated and transferred from the substrate (Figure 6A, right), in full agreement with the concept that the mechanism of removal is dominated by the mobility of the liquid overlayer on top of which the adsorbed organic or biological material rests, as observed in previous investigations of immobilized liquid layers as anti-fouling surfaces. In addition, no difference in release time was observed for glutaraldehyde-fixed cells versus living cells.

[0105] Sheets were also immunostained for fibronectin, f-actin, and nuclei to visualize their cellular organization and structure compared to controls. The morphology of the cell sheets did not appear to change after removal from the PDMS surfaces demonstrated by Figure 6B, and continued to closely mimic that of a confluent cell layer on TCPS. Further imaging of stained sheets showed no change in morphology before and after transfer, as illustrated by comparison of cell sheets before (Figure 9A) and after (Figure 9B) cell sheet transfer. Confocal images of cell sheets grown on infused PDMS were taken before removal and after removal. F-actin appears green, fibronectin – red, and the nuclei – blue. All three components were found to be present both before and after removal, and the organization and morphology of the sheet overall appeared unchanged. Furthermore, the fibronectin layer can clearly be seen underneath the cells after removal, in agreement with the results from Figure 6A. Cell sheet viability 1 h after transfer was determined using a live/dead staining assay. FN-coated PDMS and untreated TCPS were also stained as positive controls. Visually, there was no difference observed between the sheet after transfer and the TCPS controls.

Quantitatively, the cell sheet showed 97.2% ($\pm 0.8\%$) live cells, compared to 98.3% ($\pm 0.3\%$) and 98.7% ($\pm 0.5\%$) for the fibronectin-coated PDMS and TCPS, respectively as shown by the bar graph in Figure 6C.

[0106] In order to confirm that the cells within the sheet could continue to grow, we monitored the sheets for up to 5 days after transfer onto fresh unmodified TCPS surfaces. Normal growth and proliferation was observed. Figure 7A shows a transferred cell sheet with a large gap immediately after transfer, after 24 h and a change of medium, and after 48 h and another change of the medium. The cells of the sheet have grown completely over the entire surface area after just 24 h. However, we observed that in using this method small oil droplets were sometimes present on the sheets immediately after transfer. These droplets could be washed away through normal changes of growth medium and did not appear to disrupt cell growth.

[0107] To further investigate the localization of the excess silicone oil on the cell sheet after transfer, we used a silicone oil-soluble fluorescent dye to perform the cell release (Fig. 3b). The leftmost image of Figure 7B shows that the dyed oil has indeed spread evenly across the surface and appears to be present on the edges of the sheet. The center image shows groups of cells that were transferred, and are coated with the dyed oil. However, after a change of the medium the amount of dyed oil on the cell surface decreases significantly (rightmost image).

[0108] In summary, we have demonstrated how oil-infused polymers can be used as cell sheet release surfaces for MSCs. A thin layer of PDMS was used to coat the bottom of a TCPS plate, then infused with silicone oil to produce the substrate. After treatment to remove excess silicone oil, the surface of the infused layer was coated with fibronectin to encourage cell growth and proliferation. Mouse MSCs were then seeded on the surface. After the cells had grown to confluency or near-confluency, the sheet was released from the surface by

introducing an excess amount of silicone oil in a pool underneath the cell sheet by way of a syringe. The excess oil was able to slide underneath the cell sheet, releasing it from the surface. The released cell sheet could then be transferred to a new surface by means of a sheet of filter paper. Immunostaining of the cell sheets post-transfer showed no significant differences in the amount of fibronectin or f-actin per nucleus compared to cells grown on tissue culture polystyrene.

[0109] We found that initial removal of excess silicone oil and deposition of a FN layer were essential to allowing the cells to grow on infused polymers. It is well known that FN is an abundant ECM protein with adhesion promoting properties. Once a FN layer was added to the surface growth, proliferation proceeded to a level equal to that of TCPS. Previous work on infused polymers has shown that they naturally resist the adhesion of proteins, adding to their value as anti-fouling materials. For this reason, the initial removal of the excess silicone oil with washing and blotting was necessary to permit the FN layer to weakly attach to the substrate and not wash away before the cell medium could be introduced.

[0110] While we did find that some silicone oil is transferred with the cell sheet upon removal (Figures 7A and 7B), we did not see any signs of toxicity with the MSCs used here. In fact, the cell sheet was observed to grow right up to the base of the excess silicone droplets (Figure 7B). These results are not surprising, as extensive investigations of silicones for implantation into the human body have shown no direct cytotoxic effects. Moreover, our results show that the oil transferred with the cells can be washed away, resulting in a “healthy”, oil-free cell construct. The fact that the oil droplets and even the layer coating the cells could be so easily removed may explain the lack of problems with growth and proliferation using this method.

[0111] The approach to cell sheet-release surfaces described here adds to the library of methods for producing intact, free-standing cell sheets or even individual cells or protein

constructs. Using an immobilized liquid layer to create a surface with controllable adhesion based on the thickness of the layer is a completely different approach than previously developed technologies which rely on responsive polymers to change configuration upon input of a particular stimulus. This may offer some important benefits, including application to cell types which are temperature, electro-, or photo-sensitive. Furthermore, the use of PDMS as a substrate permits easy benchtop fabrication of these materials with minimal training, and the relatively short time needed to release an entire sheet (5 ± 1.9 min) may permit more high-throughput applications than were previously possible with technologies that often required 40 min or more. However, it should also be mentioned that our method, as currently described, does require physical movement of the excess oil around the plate, which if not done carefully can shred the cell sheet or result in the oil floating to the surface of the culture medium rather than remaining between the surface and the cellular layer. Future improvements could therefore involve a more automated approach to introducing and manipulating the excess oil to reduce or eliminate user error.

[0112] The use of infused PDMS for the growth and release of intact cell sheets also promises a number of future advantages in addition to the more immediate benefits described above. First, it is well known that MSCs can be caused to differentiate into different cell types ranging from neurons to osteoblasts depending on the stiffness of the underlying substrate. Furthermore, it has already been shown that the elastic modulus of PDMS can be easily tuned for the purpose of studying cell-surface mechanics. We have shown that the tunability of the elastic modulus is preserved through the infusion process. Although the values measured here, ranging between 2.5 (± 0.06) and 0.12 (± 0.03) MPa (depending on the initial base:crosslinker ratio of the uncured polymer), are currently only suited to the growth and differentiation of osteoblasts, it may be expanded to include values required for adipose or muscle growth through the use of softer PDMS matrices. Second, the low cost of the

silicone materials necessary to fabricate these surfaces and the easy benchtop fabrication of the substrates may make this approach more appealing for high-volume or proof-of-principle tests where speed and low cost are considerations. Finally, the ability to mold PDMS substrate into arbitrary non-planar shapes may open doors to the use of more complex geometries or the incorporation of surface patterns to produce interesting 3D cell fabrics. Such benefits may serve to facilitate the further development and use of cell sheet engineering.

[0113] Unless otherwise defined, used or characterized herein, terms that are used herein (including technical and scientific terms) are to be interpreted as having a meaning that is consistent with their accepted meaning in the context of the relevant art and are not to be interpreted in an idealized or overly formal sense unless expressly so defined herein. For example, if a particular composition is referenced, the composition may be substantially, though not perfectly pure, as practical and imperfect realities may apply; e.g., the potential presence of at least trace impurities (e.g., at less than 1 or 2%) can be understood as being within the scope of the description; likewise, if a particular shape is referenced, the shape is intended to include imperfect variations from ideal shapes, e.g., due to manufacturing tolerances. Percentages or concentrations expressed herein can represent either by weight or by volume.

[0114] Although the terms, first, second, third, etc., may be used herein to describe various elements, these elements are not to be limited by these terms. These terms are simply used to distinguish one element from another. Thus, a first element, discussed below, could be termed a second element without departing from the teachings of the exemplary embodiments. Spatially relative terms, such as “above,” “below,” “left,” “right,” “in front,” “behind,” and the like, may be used herein for ease of description to describe the relationship of one element to another element, as illustrated in the figures. It will be understood that the

spatially relative terms, as well as the illustrated configurations, are intended to encompass different orientations of the apparatus in use or operation in addition to the orientations described herein and depicted in the figures. For example, if the apparatus in the figures is turned over, elements described as “below” or “beneath” other elements or features would then be oriented “above” the other elements or features. Thus, the exemplary term, “above,” may encompass both an orientation of above and below. The apparatus may be otherwise oriented (e.g., rotated 90 degrees or at other orientations) and the spatially relative descriptors used herein interpreted accordingly. Further still, in this disclosure, when an element is referred to as being “on,” “connected to,” “coupled to,” “in contact with,” etc., another element, it may be directly on, connected to, coupled to, or in contact with the other element or intervening elements may be present unless otherwise specified.

[0115] The terminology used herein is for the purpose of describing particular embodiments and is not intended to be limiting of exemplary embodiments. As used herein, singular forms, such as “a” and “an,” are intended to include the plural forms as well, unless the context indicates otherwise.

[0116] It will be appreciated that while a particular sequence of steps has been shown and described for purposes of explanation, the sequence may be varied in certain respects, or the steps may be combined, while still obtaining the desired configuration. Additionally, modifications to the disclosed embodiment and the invention as claimed are possible and within the scope of this disclosed invention.

[0117] The foregoing and other features and advantages of various aspects of the invention(s) will be apparent from the following, more-particular description of various concepts and specific embodiments within the broader bounds of the invention(s). Various aspects of the subject matter introduced above and discussed in greater detail below may be implemented in any of numerous ways, as the subject matter is not limited to any particular

manner of implementation. Examples of specific implementations and applications are provided primarily for illustrative purposes.

CLAIMS

What is claimed is:

1. A system for the growth and detachment of cells, comprising:
a biocompatible substrate infused with a water-immiscible liquid-, wherein the water-immiscible-liquid infused within the substrate is in an amount sufficient to form a slippery lubricating layer on a surface of the substrate; and
a cell adhesion agent deposited on the substrate.
2. The system of claim 1, wherein the cell adhesion agent is selected from the group of ligands, hormones, growth factors, proteins, and cell behavior regulators or mixtures thereof.
3. The system of claim 1, wherein the cell adhesion agent comprises an extracellular matrix protein.
4. The system of claim 1, wherein the cell adhesion agent comprises an fibronectin.
5. The system of any of claims 1-4, wherein the cell adhesion layer is directly disposed on the substrate.
6. The system of claim 1, wherein the slippery lubricating layer comprises an immobilized liquid layer at the surface of the substrate.
7. The system of any preceding claim, wherein the substrate comprises a polymer.
8. The system of claim 7 wherein the substrate is a polymer sheet.
9. The system of claim 7, wherein the substrate is a molded polymer, and optionally can include surface patterns.
10. The system of claim 9, wherein the substrate is molded in an irregular shape or pattern.
11. The system of claim 9, wherein the water immiscible-liquid-infused substrate is in the shape of a scaffold for tissue engineering.

12. The system of claim 1 or 2, wherein the substrate comprises a porous solid.
13. The system of claim 1 or 2, wherein the water immiscible-liquid comprises an oil.
14. The system of claim 13, wherein the oil is a silicone or perfluorocarbon oil.
15. The system of claim any preceding claim, further comprising a culture plate for holding the biocompatible water immiscible-liquid-infused substrate.
16. The system of claim 14, wherein the biocompatible water immiscible-liquid-infused substrate coats the culture plate.
17. The system of claim 14, wherein the culture plate comprises a well-plate or tissue culture plastic.
18. The system of claim 14, further comprising a culture medium selected to support the growth of cells on the biocompatible water immiscible-liquid-infused substrate contained within the culture plate.
19. The system of claim 1 or 2, wherein the substrate is polydimethylsiloxane and the water immiscible-liquid is a silicone oil.
20. The system of claim 1, further comprising a cell.
21. The system of claim 20, wherein the cell is selected from ligament cells, hepatocytes, epithelial cells, myocardial cells, and/or mesenchymal stem cells.
22. A kit for the growth and detachment of cells, comprising:
 - a culture plate comprising at least one well; and
 - a curable polymer precursor, capable of forming a polymer sheet on curing;
 - a water immiscible-liquid selected for its ability to infuse into a cured polymer sheet; wherein the polymer sheet and the water immiscible-liquid are capable of forming a biocompatible water-immiscible-liquid-infused substrate adapted for culturing cells coating the at least one well, wherein the water

immiscible-liquid absorbed within the polymer is in an amount sufficient to form a slippery lubricating layer on a surface of the liquid-infused substrate.

23. The kit of claim 14, further comprising a cell adhesion agent deposited on the water immiscible-liquid-infused substrate.

24. The kit of claim 15, wherein the cell adhesion agent comprises fibronectin.

25. A cell system comprising:

a biocompatible water immiscible-liquid-infused substrate adapted for culturing cells, wherein the water-immiscible liquid absorbed within the substrate in an amount sufficient to form a slippery lubricating layer on a surface of the water immiscible-liquid-infused substrate; and

a confluent or near-confluent layer of cells adhered to the surface of biocompatible water immiscible-liquid-infused substrate.

26. The cell system of claim 17, further comprising a layer of cell adhesion agent disposed between the substrate and the cells.

27. The cell system of claim 17, wherein the substrate is molded in an irregular shape or pattern..

28. A method of cell sheet growth and detachment, comprising:

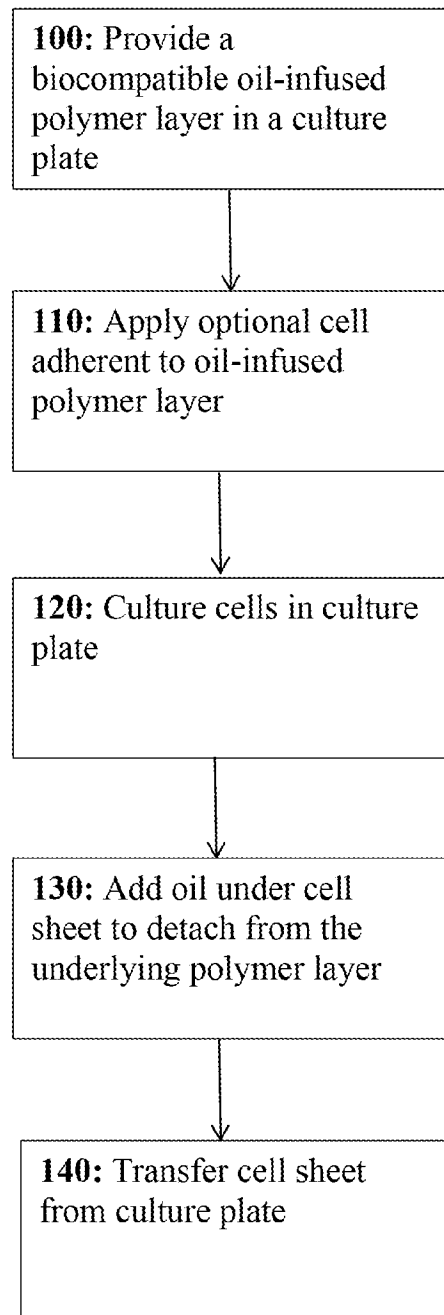
providing a system for the growth and detachment of cells according to any one of claims 1-21;

growing a cell of interest on the system to confluency or near confluency to form a cell sheet;

once confluency or near-confluency is reached, introducing a volume of oil underneath the cell sheet causing the cell sheet to separate from the surface while remaining intact.

29. The method of claim 28, further comprising transferring the cell sheet onto a new surface.
30. The method of claim 28, wherein cells are selected from the group consisting of ligament cells hepatocytes, epithelial cells, myocardial cells, mesenchymal stem cells.
31. The method of claim 28, wherein the water immiscible-liquid-infused substrate comprises an elastomeric polymer swollen with an oil.
32. The method of claim 31, wherein excess oil is removed from the polymer surface before application of the cell adherent agent.
33. The method of claim 28, wherein the cell adherent agent is a extracellular matrix protein.
34. The method of claim 28, wherein the cells are grown to at least 75% confluence, or at least 80% confluence, or at least 85% confluence, or at least 90% confluence.
35. A method of sheet growth and detachment comprising:
providing a biocompatible substrate infused with a water-immiscible liquid, wherein the water-immiscible liquid infused within the substrate is in an amount sufficient to form a slippery lubricating layer on a surface of the substrate;
forming a layer or film of a biomolecule on the substrate; and
introducing a volume of liquid underneath the layer or film causing the layer or film to separate from the surface while remaining intact.
36. The method of claim 35, wherein the biological molecule is selected from proteins, extracellular matrices and polysaccharides.

FIG. 1



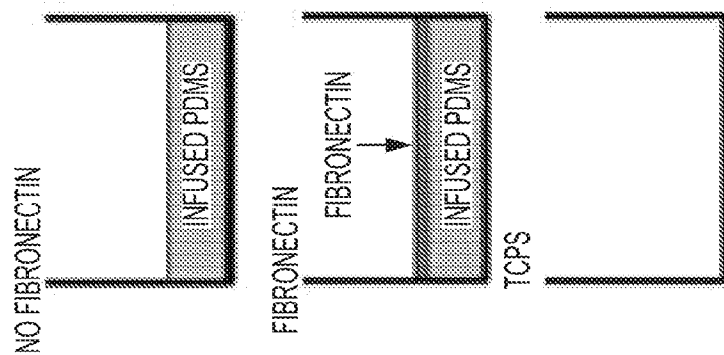


FIG. 2A

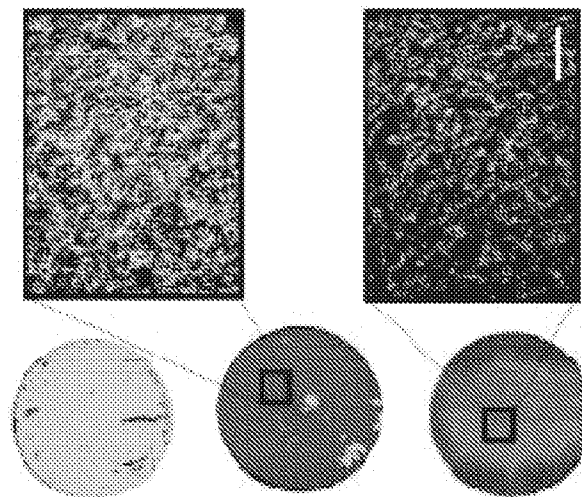


FIG. 2B

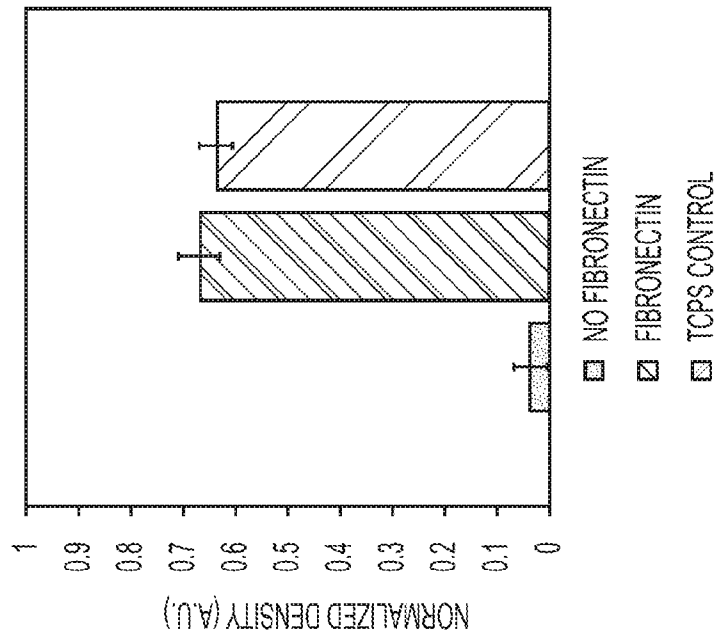


FIG. 2C

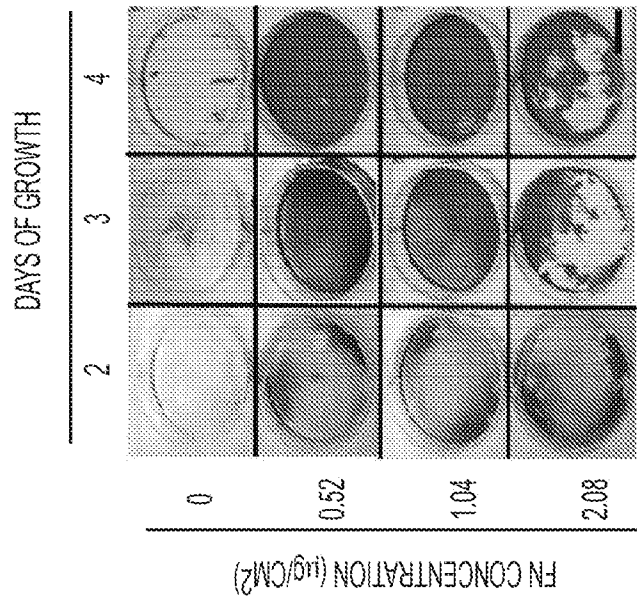


FIG. 3B

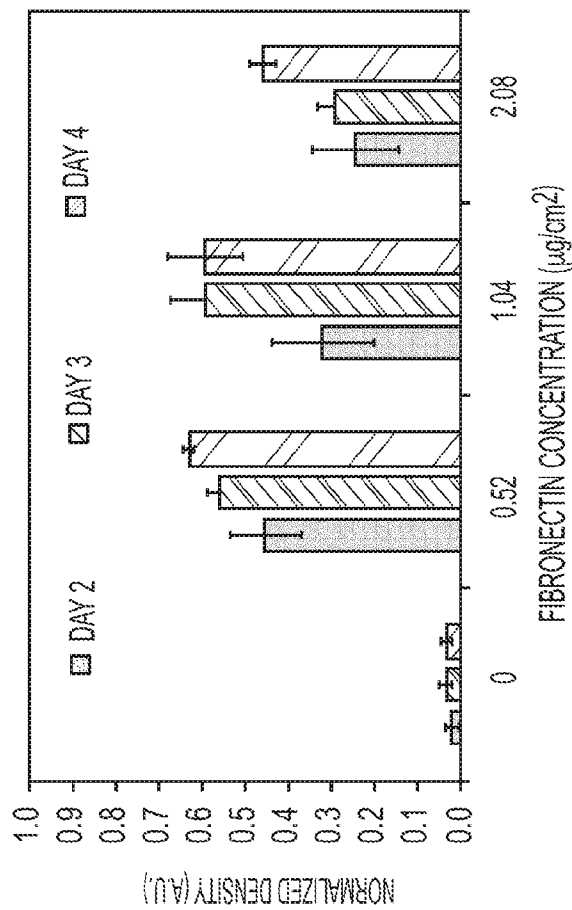


FIG. 3A

FIG. 4A

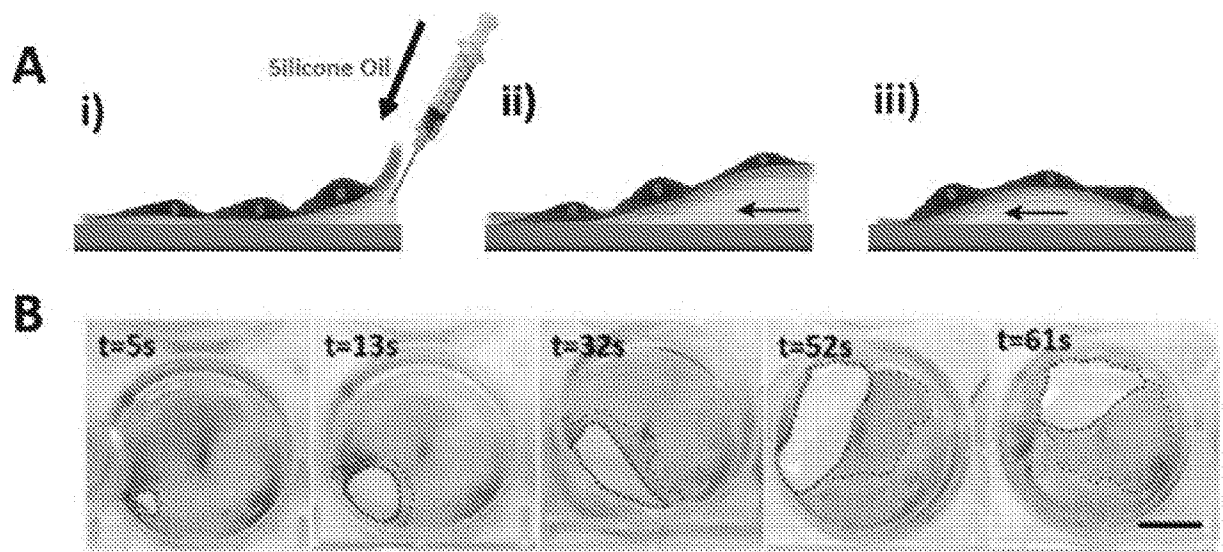
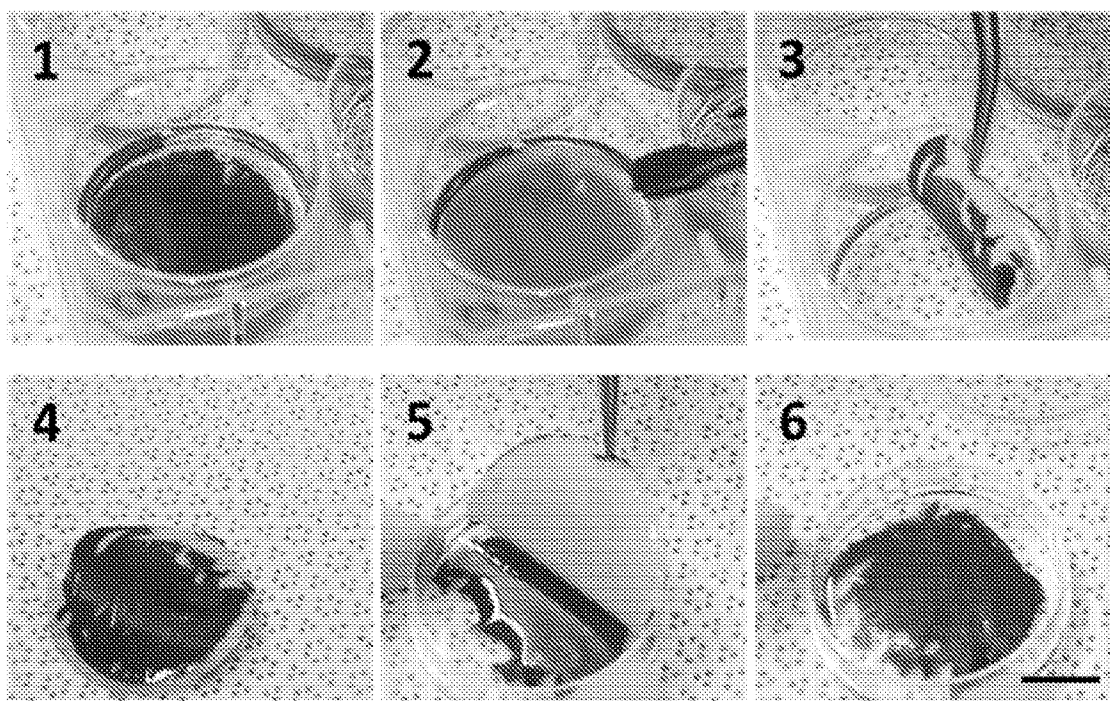


FIG. 4B

FIG. 5



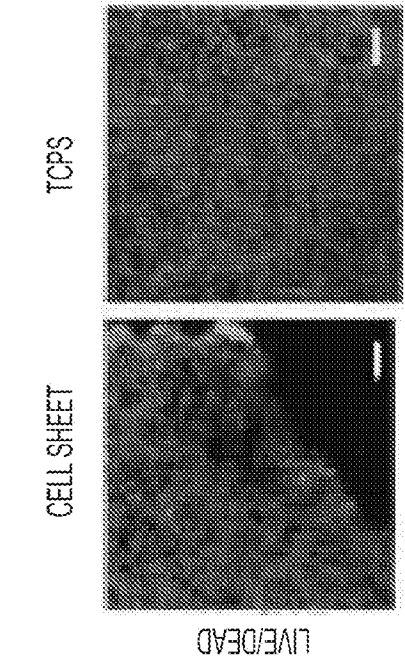


FIG. 6C

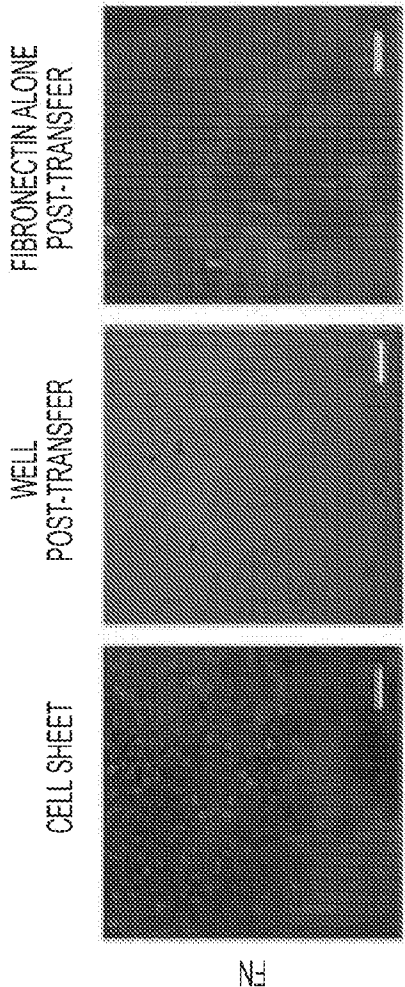
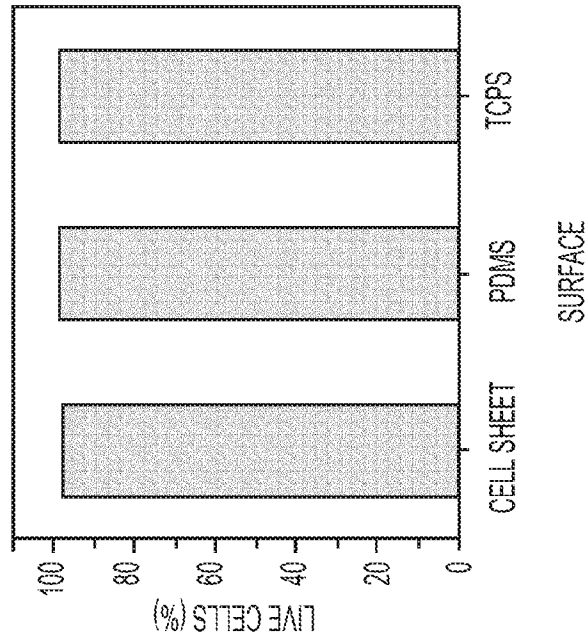


FIG. 6A

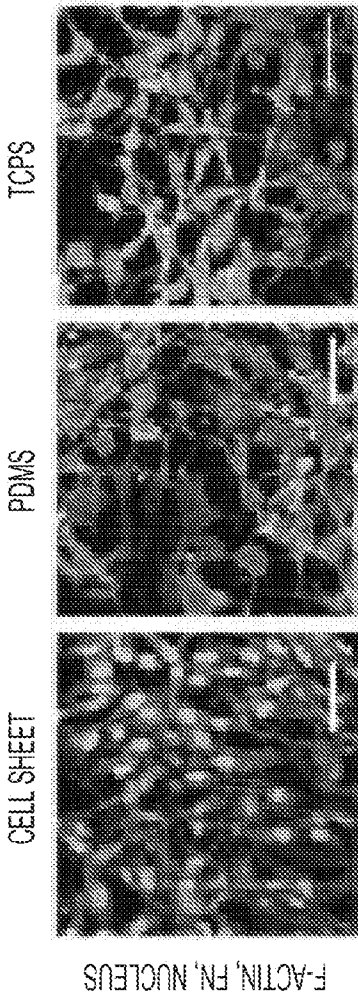


FIG. 6B

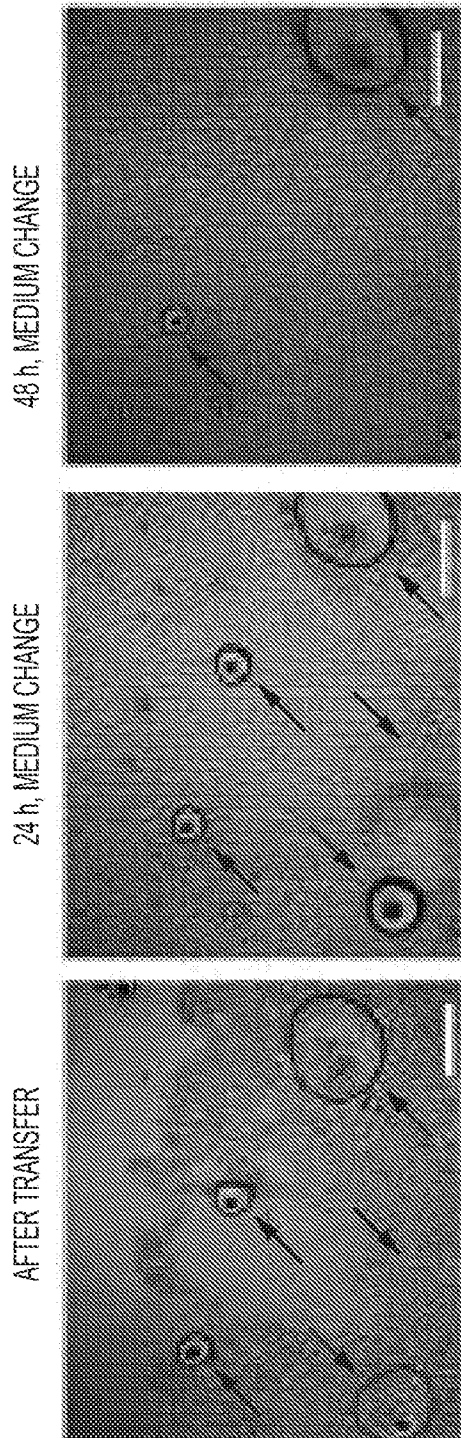


FIG. 7A

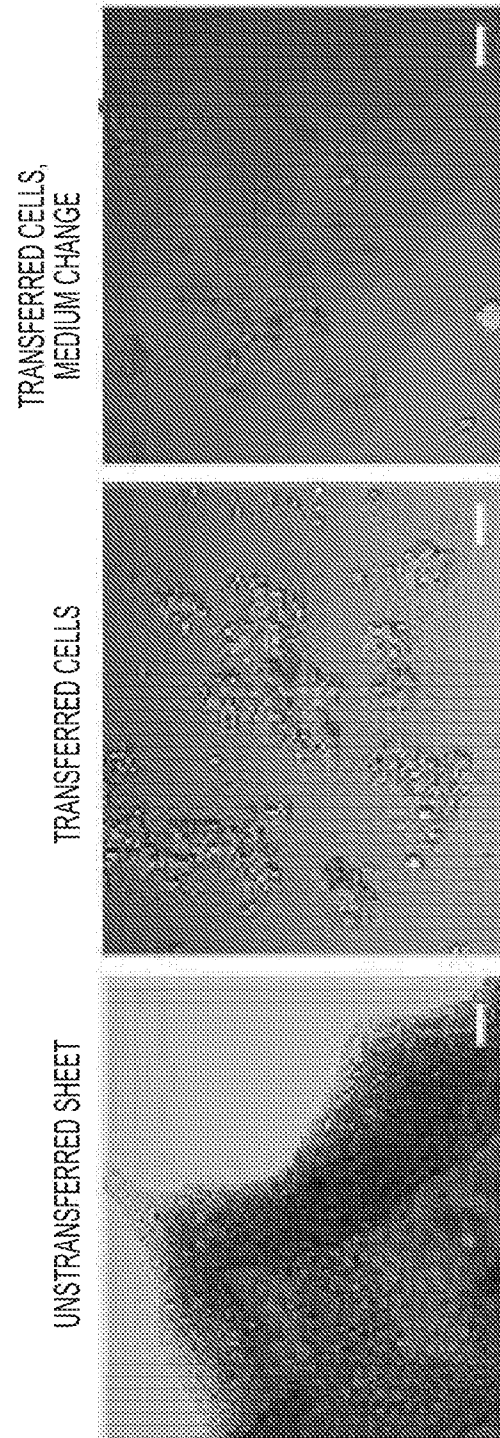
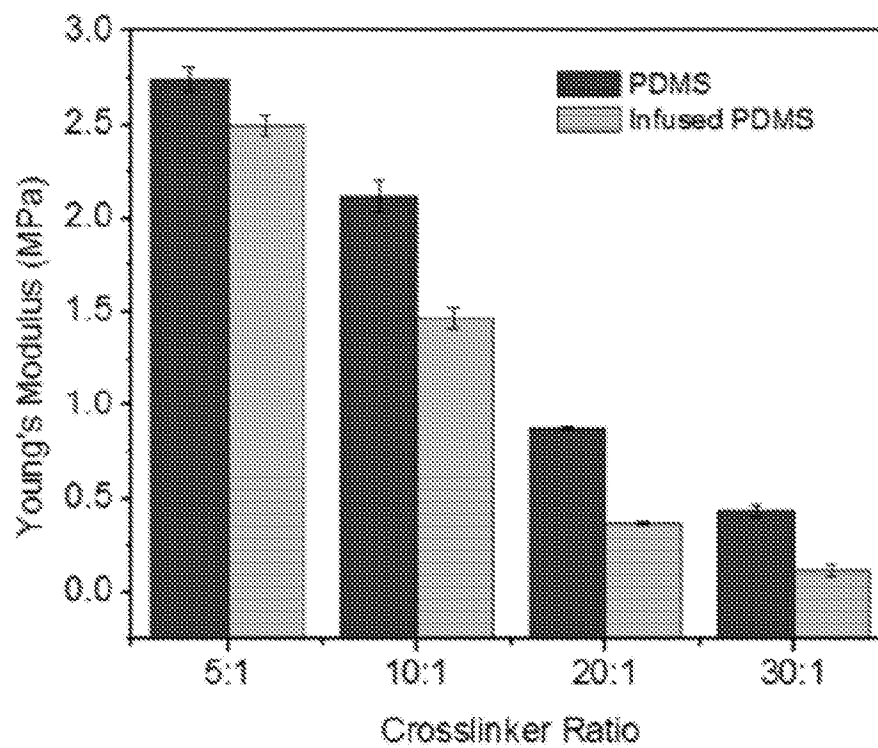
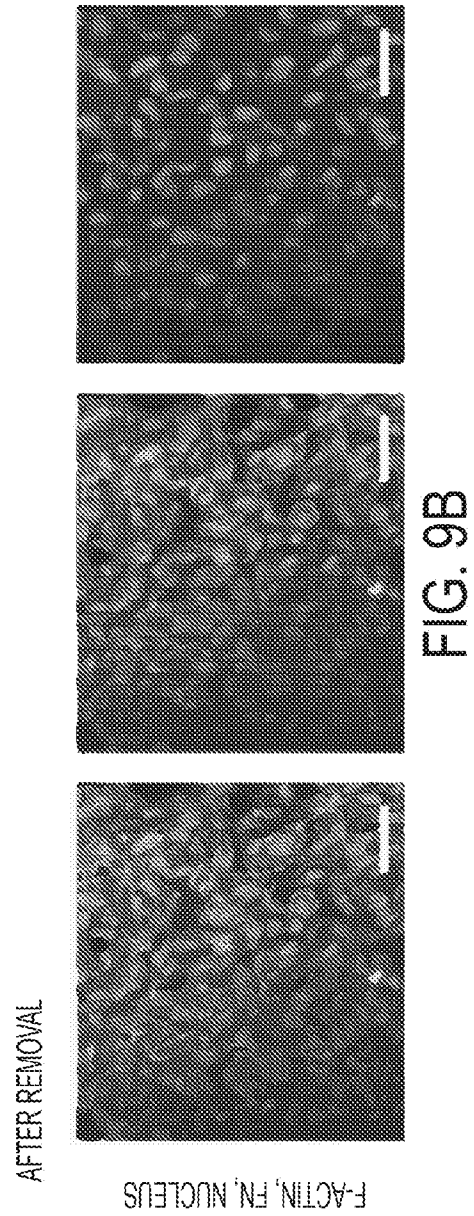
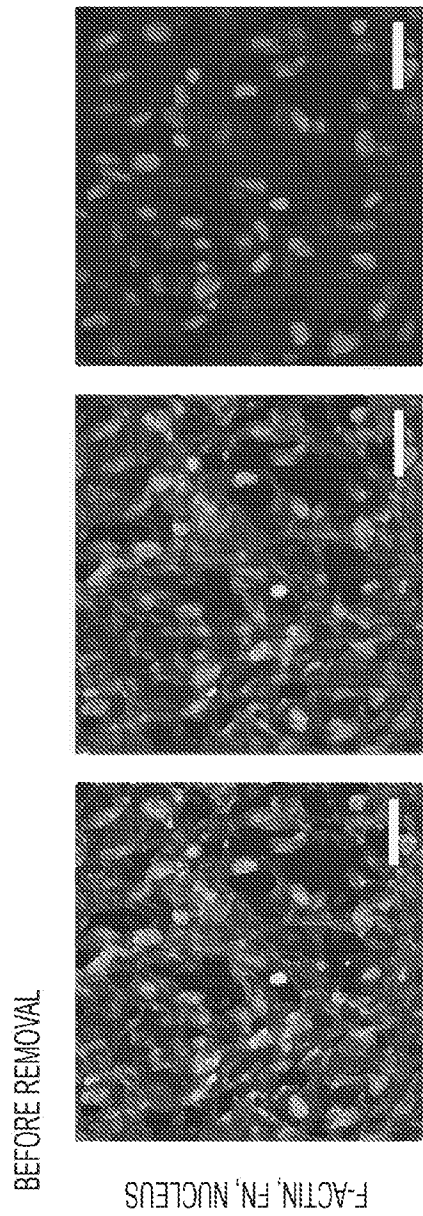


FIG. 7B

FIG. 8





INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/31480

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - B05D 5/08, 3/10; C10M 105/76; A61K 38/39; C07K 14/78 (2016.01)

CPC - B05D 5/08; C10M 105/76

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)

IPC(8) - B05D 5/08, 3/10; C10M 105/76; A61K 38/39; C07K 14/78 (2016.01);

CPC - B05D 5/08; C10M 105/76, 2229/02; C12N 2533/30, 2533/50, 2533/52, 2533/90; C10N 2250/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA); Google Scholar; EBSCO; PubMed;
 grow, culture, detach, remove, transport, water-immiscible, oil, SLIPS, lubricate, slick, pate, substrate, surface, cell adhesion, ligand,
 hormone, extracellular matrix, protein, fibronectin, collagen

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2014/0187666 A1 (AIZENBERG, J et al.) 03 July 2014; paragraphs [0107]-[0108], [0115]-[0116], [0127], [0129], [0158], [0188], [0218], [0231], [0274]-[0276]; figure 7A	1-4, 5/1-4, 6, 12/1-2, 13/1-2, 20, 22-27 --- 14, 19/1-2, 21, 35-36
Y	WO 2014/012080 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 16 January 2014; paragraphs [0158], [0211], [0239]	14, 19/1-2, 35-36
Y	US 2010/0221303 A1 (LE VISAGE, C et al.) 02 September 2010; paragraphs [0068], [0074]	21
A	US 6,737,074 B2 (MARX, G et al.) 18 May 2004; entire document	1-4, 5/1-4, 6, 12/1-2, 13/1-2, 14, 19/1-2, 20-27, 35-36
A	US 2014/0087173 A1 (KARLSRUHER INSTITUT FUR TECHNOLOGIE) 27 March 2014; entire document	1-4, 5/1-4, 6, 12/1-2, 13/1-2, 14, 19/1-2, 20-27, 35-36

☐ 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 another 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

13 July 2016 (13.07.2016)

Date of mailing of the international search report

12 AUG 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/31480

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7-11, 15-18, 28-34
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.